05/31-06/03 Preliminary yeast catalase assay

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-05-31 to 2016-06-03

TUESDAY, 5/31/16

We will test if yeast cells have to be lysed to test theis catalase activity. This will be important to know if we want to use catalase activity as a sensor.

☒ Yeast liquid culture

WEDNESDAY, 6/1/16

We will have E. coli as two controls to see a proper catalase reaction

- one control as a suspension (1 ml H2O + colonies)
- one control just cells

☒ Yeast cell lysis

Catalase activity of the yeast will be tested in several time points

- after incubation in the liquid growth medium
- after addition of first lysis buffer (A1) and lysing with glass beads
- after addition of second lysis buffer (A2) (in addition to the assay)

☒ Catalase activity assay

Results

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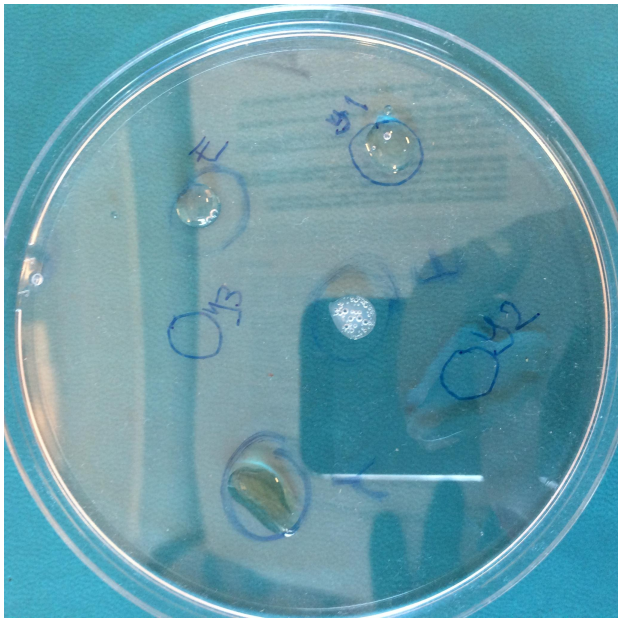

Table1

	A	B	C
1	Code in the picture	Sample	Catalase activity
2	E (upper)	E. coli in H2O	+
3	E (middle)	plain E. coli	+++
4	Y	after growth	-
5	Y1	after A1	+
6	Y2	after A2	-
7	Y3	nothing	

To improve

- test with TritonX-100
- test with various H2O2 concentrations and volumes

THURSDAY, 6/2/16

For further test assays, four liquid cultures

☒ Yeast liquid culture

Also, we strieked a new plate for our own use from the yeast provided by Mari
One colony, YPD plate

FRIDAY, 6/3/16

- We used liquid culture of S. cerevisiae W303a strain, that had
- Measured OD600 from each of four sample

Absorbance measurements

Table2

	A	B	C	D
1	tube	dilution	OD600	real, OD600
2	1	1:10	0.654	6.54
3	2	1:10	0.688	6.88
4	3	1:10	0.669	6.69
5	4	1:20	0.558	11.16

Catalase activity measurements

- for all measurements we used 30% H2O2 and 1% TritonX-100

Table3

	A	B	C	D	E	F	G
1	number on plate	lysed/intact cells	V(cells)/ul	V(TritonX-100)/ul	V(H2O2)/ul	tube	reagents ra (S:T:H)
2	1	intact	20	0	20	2	1:0:1
3	2	intact	20	20	20	2	1:1:1
4	3	lysed	20	0	20	2	1:0:1
5	4	lysed	20	20	20	2	1:1:1
6	5	lysed	10	10	10	2	1:1:1
7	6	lysed	10	0	10	1	1:0:1
8	7	lysed	10	10	10	1	1:1:1
9	8	lysed	20	10	10	1	2:1:1
10	9	lysed	20	10	20	1	2:1:2

- added more cells to 5 and 8 -> bubbles (a lot) -> might have something to do with the adding order

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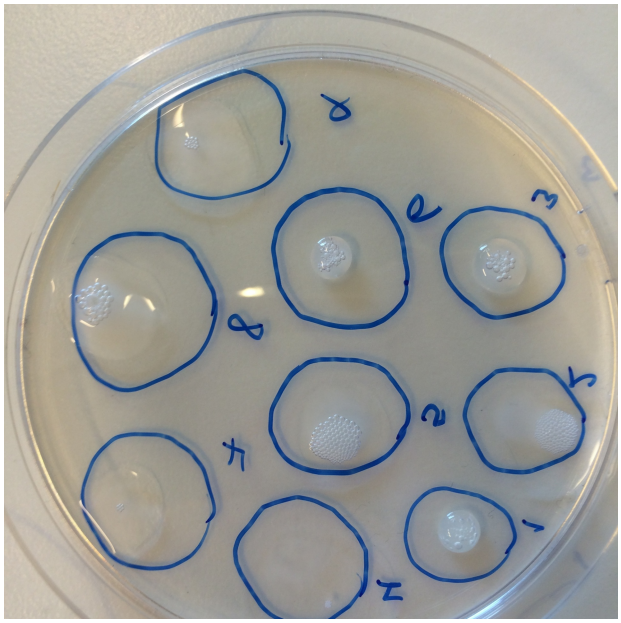
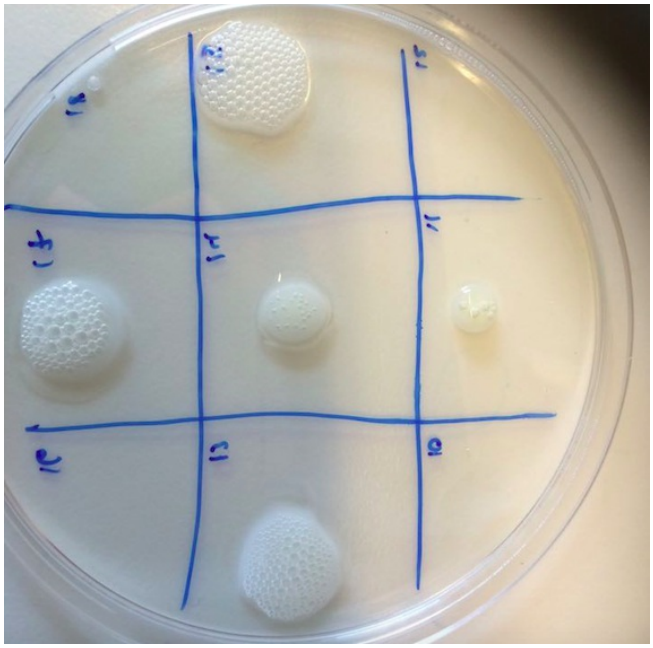


Table4

	A	B	C	D	E	F	G
1	number on plate	lysed/intact cells	V(cells)/ul	V(TritonX-100)/ul	V(H2O2)/ul	tube	reagents ra (S:T:H)
2	10	lysed	20	10	20	1	2:1:2
3	11	intact	20	0	20	4	1:0:1
4	12	intact	20	10	20	4	2:1:2
5	13	intact	20	10	20	3	2:1:2
6	14	intact	20	10	20	3	2:1:2
7	15	intact	20	10	20	3	2:1:2
8	16	intact	20	0	20	3	1:0:1
9	17	intact	20	10	20	3	2:1:2

benchling1.png



-notes:

- without TritonX bubbles last shorter time
- same problem when triton added first

-> best option: first cells (20ul) > then H2O2 (20ul) and TritonX right after H2O2 --> results in lots of bubbles, that last longer

06/02-07/02 Growth test

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-06-02 to 2016-07-01

THURSDAY, 6/2/16

- o/n cultures from W303 and VL3 were prepaired
- left to grow in 32°C

FRIDAY, 7/1

- OD600 was measured from both samples
- they were diluted to reach same OD600, about 0,5
    - 1:4 dilutions and again 1:4 or 1:5 (vl3) were made
- measured ODs and dilutions are presented in table 1

Table1

	A	B	C	D	E
1		OD600	OD600		
2		no dilution	1:4	1:16	1:20
3	W303	2,102	1,17	**0,651**	
4	VL3	2,309	1,761	0,784	**0,697**

-100ul of chosen W303 and VL3 dilutions (bolded ones) were pipetted in the first column of the wells
-4 consecutive dilutions of 1:4 were made
-100ul of toxin solution with varying concentrations were pipetted into wells among the table 2
-in first row 2% MeOH was added instead of toxin (as a negative control)
-5 ul of each sample was pipetted in YPD plate and left to grow in 30°C

Table3

	A	B	C	D	E	F	G
1			1	2	3	4	
2	no toxin	A	1	1:4	1:64	1:64	1:256
3	0,1 ug/l	B	1	1:4	1:64	1:64	1:256
4	1 ug/l	C	1	1:4	1:64	1:64	1:256
5	0,01 mg/l	D	1	1:4	1:64	1:64	1:256
6	1 mg/l	E	1	1:4	1:64	1:64	1:256
7	10 mg/l	F	1	1:4	1:64	1:64	1:256

- plating was done so that cell concentration decreases to right and toxin concentration increases when going down

1.jpg

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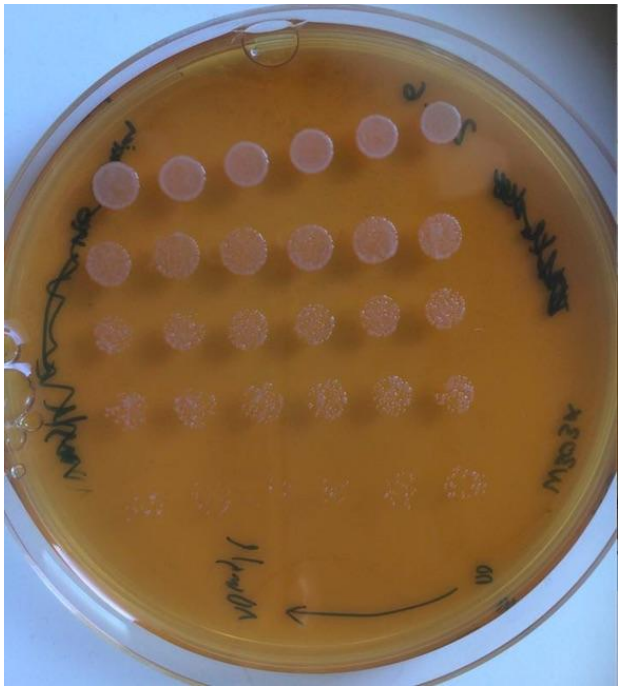


Results and interpretation:

Growth of neither of the strains hasn't been affected by the toxin much. Maybe in W303 toxin reduces growt a bit, but in VL3 there is no change.

In VL3 there are much more colonies (some are still very small so they don't show in the picture well) so could it be that even if it transports MC inside the cell, it supports it better because of it's stronger catalase response? (VL3 produced much more foam in the presence of H_2O_2.) This strong catalase response would so on protect yeast cells in stress situations, as in presence of MC. But there is more colonies also in control so maybe this is not valid way of thinking.

The control was done with methanol-water solution (2%) because all the toxins were suspended into it. But we should have done also control with only water

06/20-06/28 VL3 tests

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-06-20 to 2016-06-28

MONDAY, 6/20

Saccharomyces cerevisiae from Zymaflore, dried

- Small amount of dry VL3 to 5 ml YPD
- O/N at +30 °C, shaking at 230 rpm

TUESDAY, 6/21

Plated VL3 yeast on YPD plates

- 10, 20 and 30 ul of yeast liquid culture
  - three different volumes to make sure we have the right amount of yeast on some of the plates
- O/N at +30 °C overnight

WEDNESDAY, 6/22

- restreaked VL3 to new plates and left to grow in 37°C
- There was so much biomass that plates couldn't be left to grow another day
- 12 colonies were picked for this

THURSDAY, 6/23

- VL3 plates were moved to fridge
- W303a was plated and left to RT over Juhannus

MONDAY, 6/27

- 3 liquid cultures of 5ml were made from both VL3 and W303

TUESDAY, 6/28

- O/N cultures to 50 ml falcons (VL3 & W303)
- centrifuge 5 000 rpm, 5 min, at 21 °C
- resuspend pellets into 2 ml PBS
- measured OD600 (table 1)

Table1

	A	B	C	D
1	sample	dilution	OD600	without dilution OD600
2	VL3		2,595	2,595
3	VL3	1:10	0,844	8,440
4	W303	1:10	0,325	3,250

values for yeast culture ODs

- diluted the cell suspensions so that OD600 = about 3
- samples for catalase test in table 2

Table2

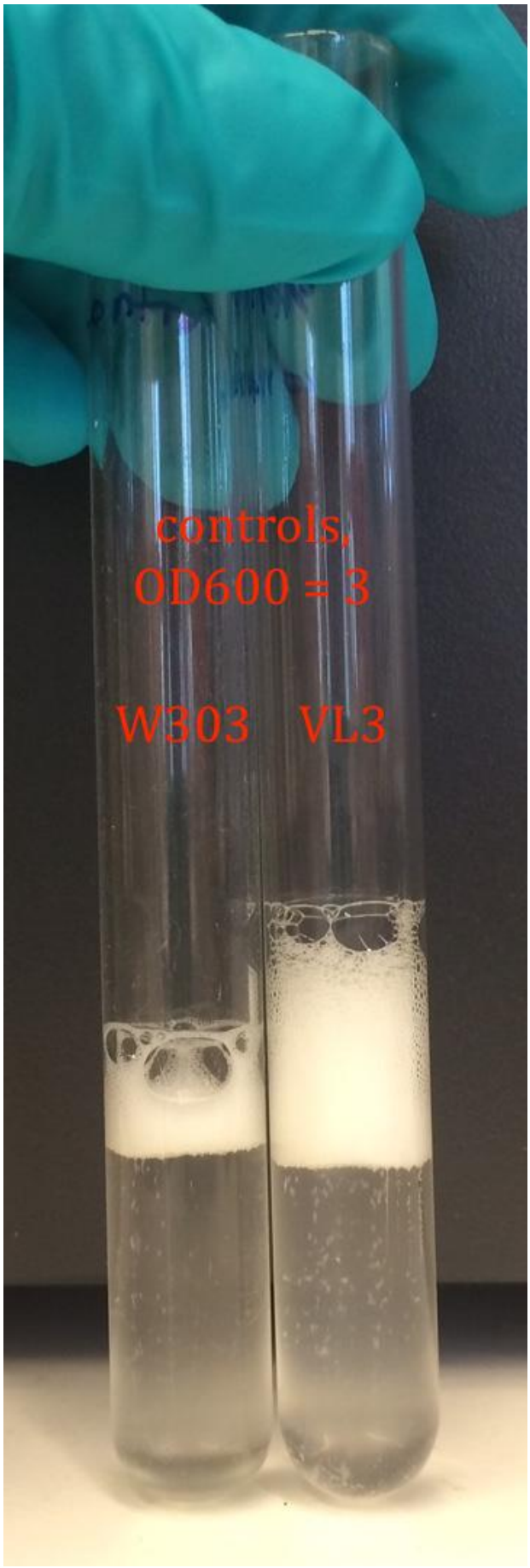
	A	B	C	D
1	Strain	OD600	c(MC, ug/L)	notes
2	W303	3	0	control
3	W303	3	1	safety limit
4	W303	3	10	
5	W303	3	1000	
6	VL3	3	0	control
7	VL3	3	1	safety limit
8	VL3	3	10	
9	VL3	3	1000	
10	VL3	8.4	0	control
11	VL3	8.4	1	safety limit
12	VL3	8.4	1000	

samples for catalase assay

- catalase test protocol
    - in sample VL3(OD=8.4, c=1000) reaction volumes were half

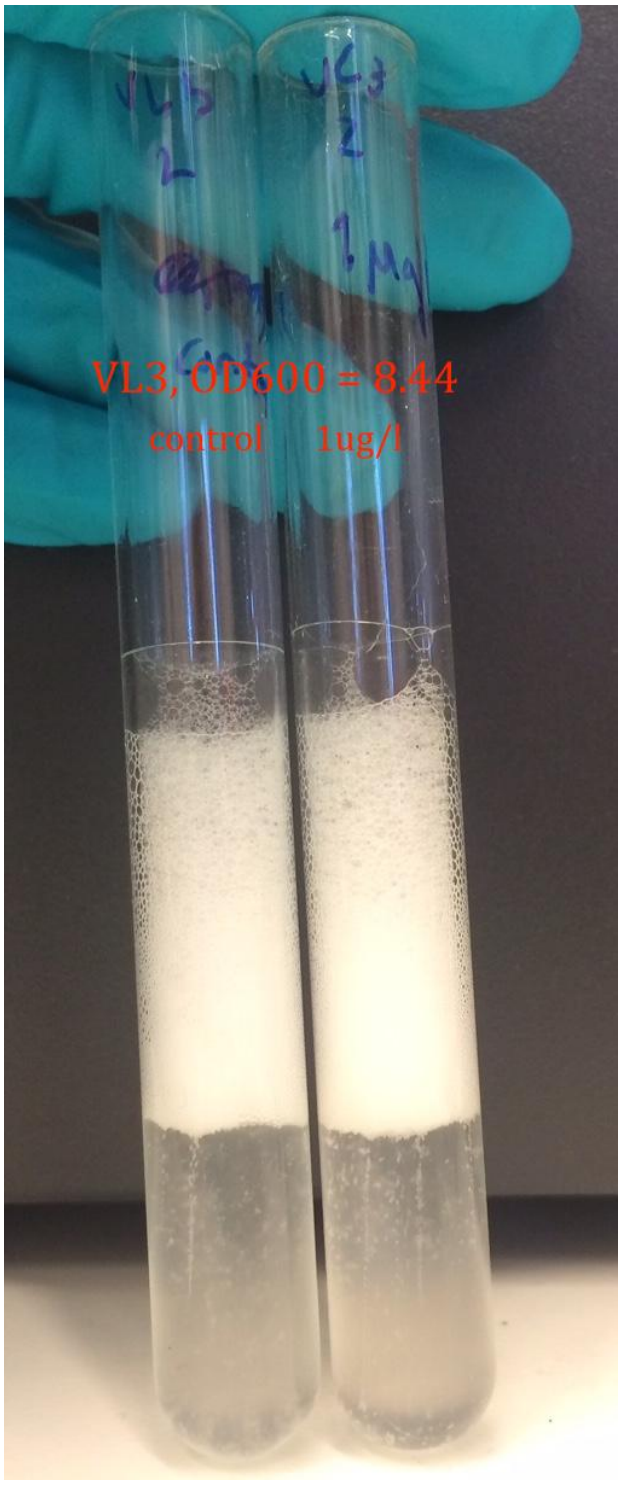
RESULTS

controls W303 VL3 catalase 280615.jpeg



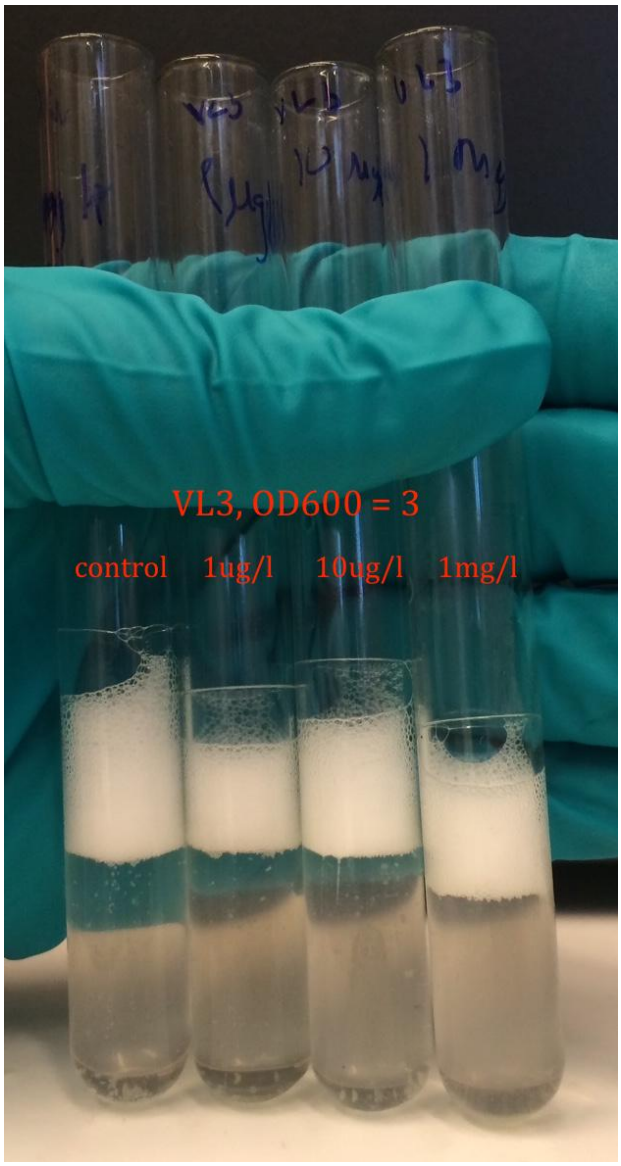
Controls from W303 and VL3 (OD=3); a clear difference in the foam formation

VL3, OD=8, 280615.jpeg



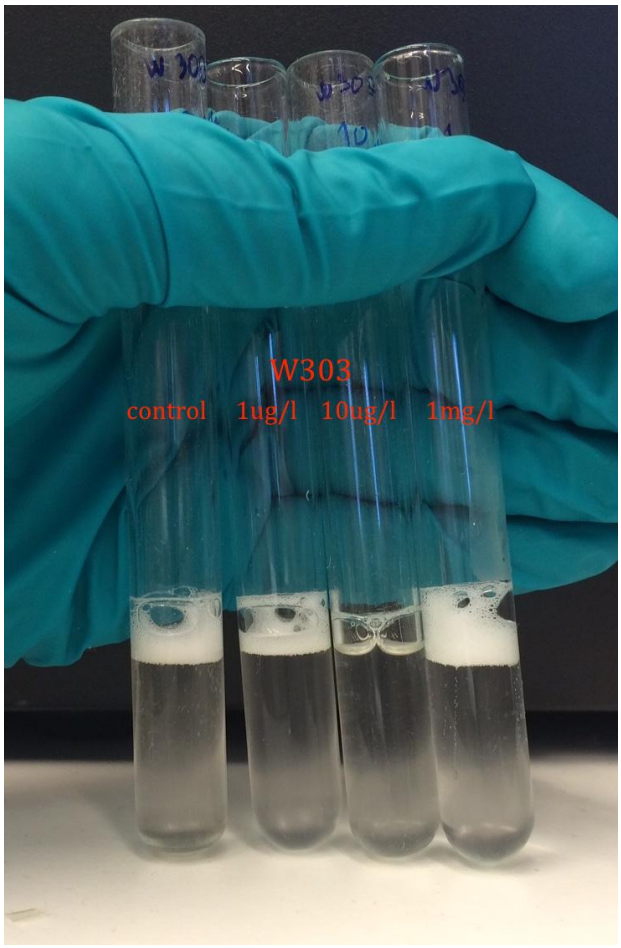
VL3, OD=8.4; control and 1ug/l; no difference in foam formation

VL3 (OD=3) catalase 280615.jpeg



VL3, OD=3; no clear difference between control and MC samples

W303 catalase 280615.jpeg



W303; no clear difference between control and MC samples

Conclusions

- VL3 has a stronger catalase response, but since no clear difference between control and other samples we can't say why this is. It might be that VL3 has a stronger stress response to MeOH -> we need to have a control with just water

07-21 pUG6 PCR linearization

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-07-21 to 2016-07-26

THURSDAY, 7/21

PCR was conducted according to the attached protocol to linearize pUG6 plasmid backbone for assembly of the transporter constructs.
Two 50 uL reactions were set up; pUG6 reactions with primer concentrations of 0.2 uM and 0.5 uM.

5 uL of each reaction was run on a 0.7 % agaose gel (using 1 uL purple loading dye 6X) to visualize results of the PCR.

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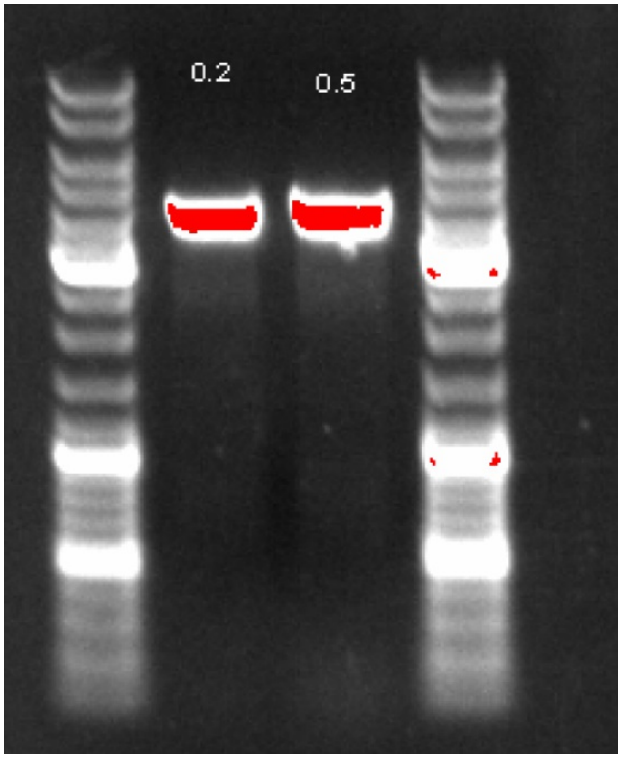

(pUG6 PCR reactions with 0.2 uM primers and 0.5 uM primers)

A band was identified around 4 kb; this corresponds to the correct product, which is 3800 bp.
Unused template was digested with DpnI; 1 uL of DpnI (NEB) was added to the raw PCR product, and the product was incubated for 1 hour at 37 C, followed by a heat-inactivation of DpnI at 80 C for 20 minutes. The resulting digested PCR product was column purified using the NucleoSpin Plasmid Gel and PCR cleanup kit (Macherey-Nagel). For the purification, solution NTI was diluted 1:5 into water, and elution was conducted in two wash steps, both involving 10 uL of elution buffer.
The concentration of the purified product was measured with Nanodrop; a concentration of 97,8 ng/uL was measured. The purified product was stored at -20 C in a tube labeled "pUG6 PCR purif. 21/7".

TUESDAY, 7/26

The purified product was checked on the gel to verify good purification.
Samples containing 0.5 uL and 1 uL of the purified PCR product were diluted with water to a volume of 2.5 uL and combined with 0.5 uL purple loading dye. Samples were run on a 0.7 % gel.

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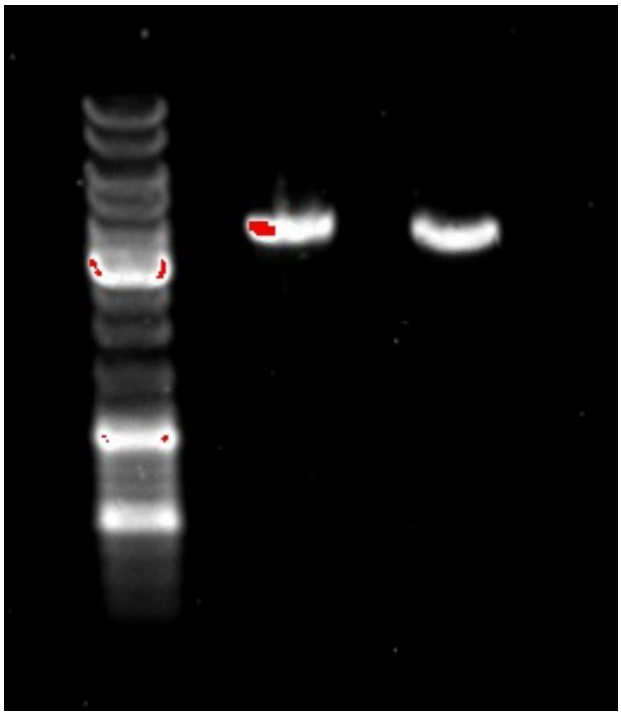

(From left:1 uL DNA and 0.5 uL DNA)

The product appeared to be well purified, so gibson assembly was proceeded to.

07-26/08-26 Transporter Assembly and Sequence Verification

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-07-26 to 2016-08-26

TUESDAY, 7/26

Gibson assembly was done to assemble transoprter plasmids pAH06 and pAH07, the transporter constucts with N-terminal strep-tag and C-terminal strep tag (QDR2I_Nstrep and QDR2I_Cstrep) and pUG6 plasmid backbone by Gibson assembly (NEBuilder) protocol, using a vector:insert molar ratio of 1:2.
The transporter gBlocks were suspended in TE buffer according to attached protocol. The PCR linearized backbone ("pUG6 PCR purif. 21/7".) was diluted 1:4 to a final volume of 8 uL, for which the concentration was checked with Nanodrop (26.6ng/uL).

Following amounts were pipetted to reaction mixture:

Table1

	A	B	C
1	total	20 ul	
2	pUG6 vector (26.6 ng/ul)	1,9 ul	50 ng
3	transporter insert (10ng/u)	4,1 ul	41 ng
4	NEBuilder Hifi master mix	10 ul	
5	sterile water	4 ul	

A positive control was made from provided pos control solution.

Mixtures were then incubated in thermocycler 50°C and 60 mins.

After incubation plasmids were transformed into provided competent cells using NEBuilder transformation protocol and grown O/N in 37°C. Both transporter constructs as well as positive control resulted in many >500 colonies. Inserts were further analysed with colony PCR.

WEDNESDAY, 7/27

Colony PCR was performed according the colony PCR (DreamTaq Green PCR master mix) protocol.

5 colonies from each plate (Nstrep and Cstrep) were used.

Primers used: forward=PS3, reverse=PS8

Mastermix (12x10ul) was prepared according the following table.

Table2

	A	B
1	DreamTaq MM	6 ul
2	PS3	2,4 ul
3	PS8	2,4 ul
4	water	55,2 ul

10 uL of mastermix was aliquotted into tubes and colony samples were picked using a sterile pipette tip.
A negative control was made with template that primers don't bind

Colony PCR products were analysed on agarose gel.

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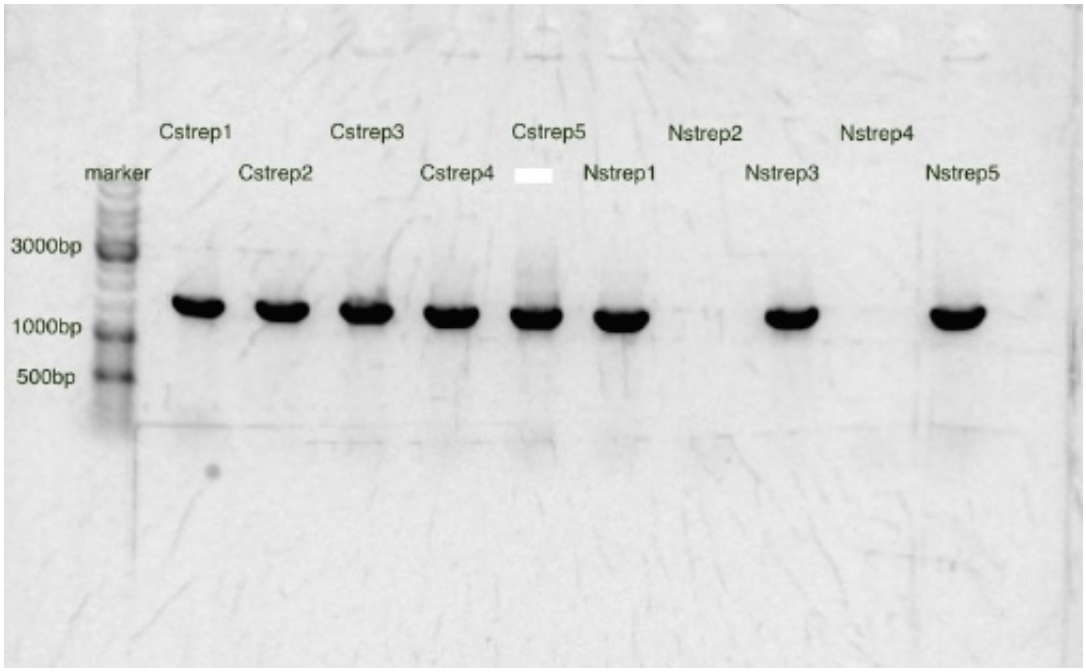

(from left; marker, QDR2I_Cstrep colonies 1-5 (pAH09), QDR2I_Nstrep colonies 1-5 (pAH08), lower row other samples,)

All Cstrep cells had correct insert in them according the colony PCR, as well as Nstrep1,3 and 5.
Liquid cultures were made from three of the samples that had the correct insert according the colony PCR.

THURSDAY, 7/28

Miniprep was done from liquid cultures. They had concentration of about 100 ng/ul. Plasmids were stored in the freezer.

Gibson assembly was done to assemble the remaining transporter constructs: pAH08, pAH09 and pAH10 (QDR_I, QDR_L and mCherry, respectively, in pUG6 backbone)

1. **mCherry**
   - 2,7 ul mCherry_QDR 10uM
   - 3,3 ul QDR2I_mCherry 10uM

- 2,7 ul pUG6 19 uM
- 1,3 ul H2O

2. **QDR_I**
   - 4,0 ul QDR1 10uM
   - 2,7 ul pUG6 19 uM
   - 1,3 ul H2O
3. **QDR_L**
   - 4,0 ul QDR1 10uM
   - 2,7 ul pUG6 19 uM
   - 1,3 ul H2O

Transformation was done according NEBuilder transformation protocol. 20 and 100ul of each transformation reaction was plated. 20 ul resulted in good number of colonies.

FRIDAY, 7/29

Colony PCR was done from transporter constructs as before (mCherry, QDR_I, QDR_L), with the same negative control. PCR products were left in fridge over weekend and analysed on monday.

MONDAY, 8/1

Colony PCR products were analysed on gel.

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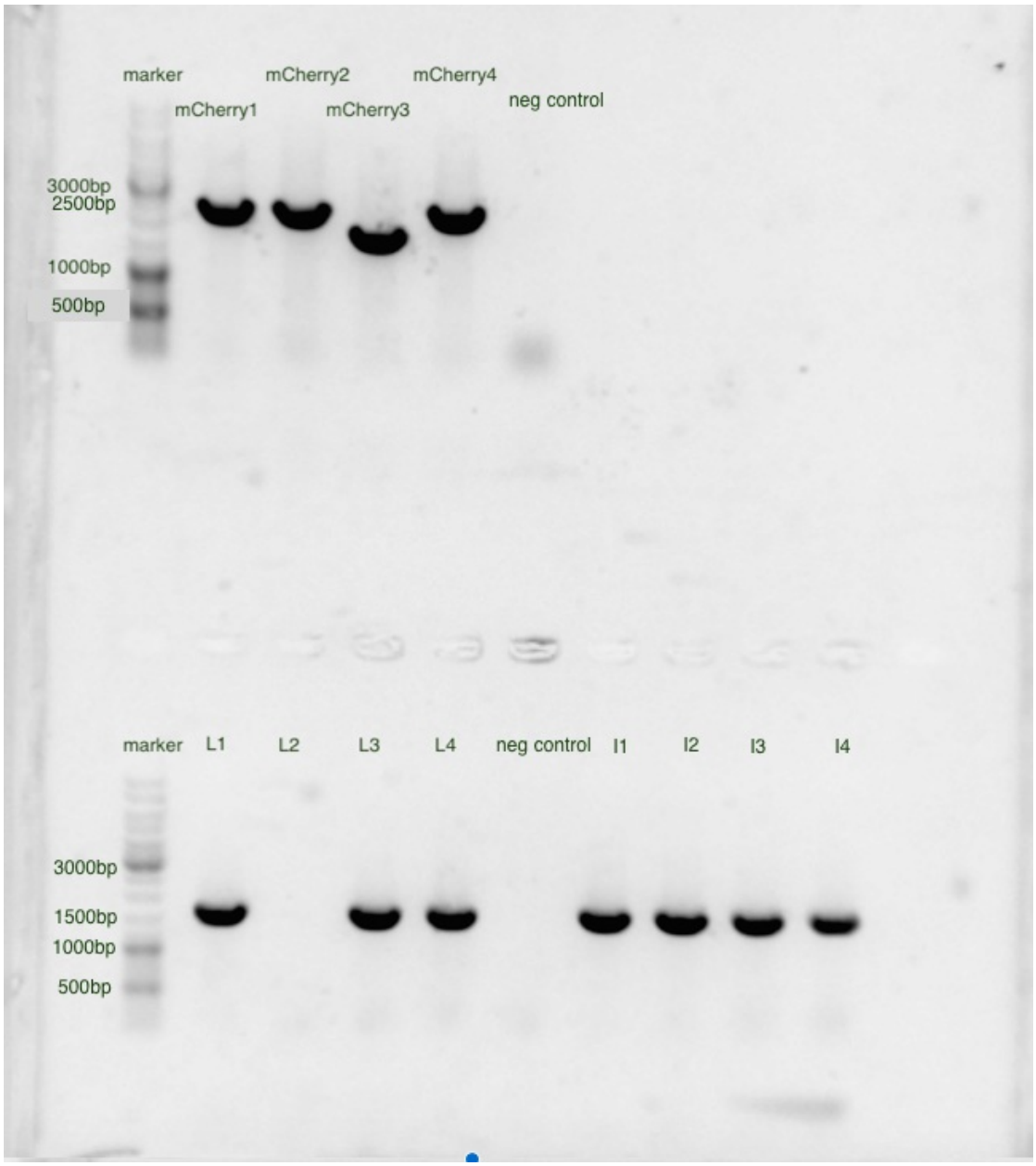

(from upper left; marker, QDR2I_mCherry colonies 1-4 (pAH10), negative control; from lower left, marker, QDR2L colonies 1-4 (pAH07), marker QDR2I colonies 1-4 (pAH06))

Liquid cultures were made from m1, m2, m3, L1, L3, L4, I1, I2 and I3.

TUESDAY, 8/2

Minipreps were made from liquid cultures and concentrations were measured with Nanodrop (60 ng/ul - 200 ng/ul). Additionally, glycerol stocks were made from the cultures by combining 500 uL of the culture with 500 uL of 50 % glycerol in a cryotube. Stocks were left in room temperature for an hour and then stored at -80 C.

A sample from one colony from each transporter construct was sent to sequencing (Eurofins, valueread). (pAH06-C2, pAH07-C1, pAH08-C1, pAH09-C2, pAH10-C1). The sent samples are in table 3:

Table3

	A	B
1	Sample	Primer
2	pAH06-C2FW	pQE for
3	pAH06-C2RV	PS8
4	pAH07-C1FW	pQE for
5	pAH07-C1RV	PS8
6	pAH08-C1FW	pQE for
7	pAH08-C1RV	PS8
8	pAH09-C2FW	pQE for
9	pAH09-C2RV	PS8
10	pAH10-C1FW	pQE for
11	pAH10-C1RV1	pEGFP_36uni
12	pAH10-C1RV2	PS8

Primers with PS8 primer were premixed samples (15 uL of template plasmid with concentration 50-100 ng/uL) and 2 uL of primer with concentration 10 uM), the others used the free eurofins primers, meaning that the samples were prepared without adding primer.

THURSDAY, 8/11

Based on sequencing results, pAH09-C2 and pAH07-C1 contained the correct insert. pAH06-C2RV didn't give a read so certainty wasn't obtained for that colony. pAH8-C1 showed a strange gap in the read, in the beginning of the sequence, so a different colony was sent for resequencing. pAH10-C1 also gave a bad read so the full sequence wasn't covered, so new samples were sent. All sent samples:

Table4

	A	B
1	pAH06-C2RV2	PS8
2	pAH08-C1FW2	pQE for
3	pAH08-C3FW	pQE for
4	pAH08-C3RV	PS8
5	pAH10-C1RV1-2	pEGFP_36uni

FRIDAY, 8/19

The following samples were again resent because clarification wasn't still obtained:

Table5

	A	B
1	pAH06-C2RV3	PS8
2	pAH10-C1RV1.3	pEGFP_36uni

This time, the samples were prepared to contain 1 % DMSO, as it was assumed that the problem with sequencing (short reads that "die" out after a couple hundred base pairs, when other times the read is up to 1000 bp) might be due to DNA secondary structure (although this would be strange, since some parallel samples produced good reads and some failed to produce any read or gave really short reads...).
In addition, based on the pAH08 result, it was noticed that the gap was also in the seond sequenced colony, and it seemed to correspond with the construct missing the N-terminal strep tag. This must mean that the wrong gBlock was used in its assembly.

FRIDAY, 8/26

Sequencing results finally confirmed that pAH06-C2 was correct, but pAH10-C1 contained a frameshift-inducing mutation in it's protein coding sequence, so it couldn't be used anymore

08-01/02 Genome Integration Cassette PCR Generation

Project: Transporter
Authors: Pihla Savola
Dates: 2016-08-01 to 2016-08-02

MONDAY, 8/1

PCR was conducted to generate the linear transporter cassette including transporter gene, KanMX selection marker, and 50 bp homology flanks on either end for genome integration. The 5' homology was pre-existing; 3' homology was created with primers.

PCR reactions were set up for strep-tagged transporter constructs, pAH08-C1 and pAH09-C2 (QDR2I_Nstrep-C1 and QDR2I_2strep-C2 in pUG6 vector).

Table1

	A	B	C
1		QDR2I_Nstrep-C1	QDR2I_Cstrep-C2
2	Phusion Hotstart II Hifi Mastermix (2X)	50 uL	50 uL
3	FW primer (PS3, 10 uM solution)	1 uL	1 uL
4	RV primer (PS4, 10 uM solution)	1 uL	1 uL
5	Template plasmid	0.4 uL (115.7ng/uL solution)	0.8 uL (66.0 ng/uL solution)
6	H2O	47.6 uL	47.2 uL
7	total	100 uL	100 uL

The PCR was carried out under the following cycyling conditions:

Table2

	A	B	C
1	98 C	2 min	
2	98 C	10 s	
3	65 C	30 s	
4	72 C	1 min	cycle to 2, x25
5	72 C	10 min	
6	4 C	hold	

PCR products were mixed with purple loading dye and run on a 0.7 % agarose gel.

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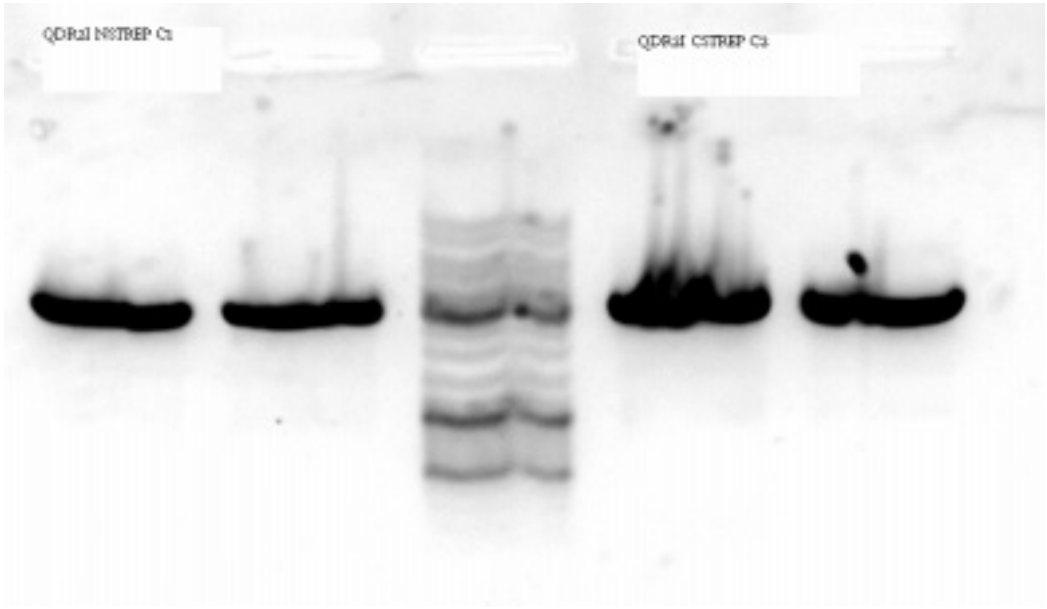

(from left; QDR2I_Nstrep-C1 in 2 wells, marker, QDR2I_Cstrep-C2)

The correct PCR product was identified around 300 bp (expected size around 3100 bp). The band was cut out, and the gel was imaged after the cutting:

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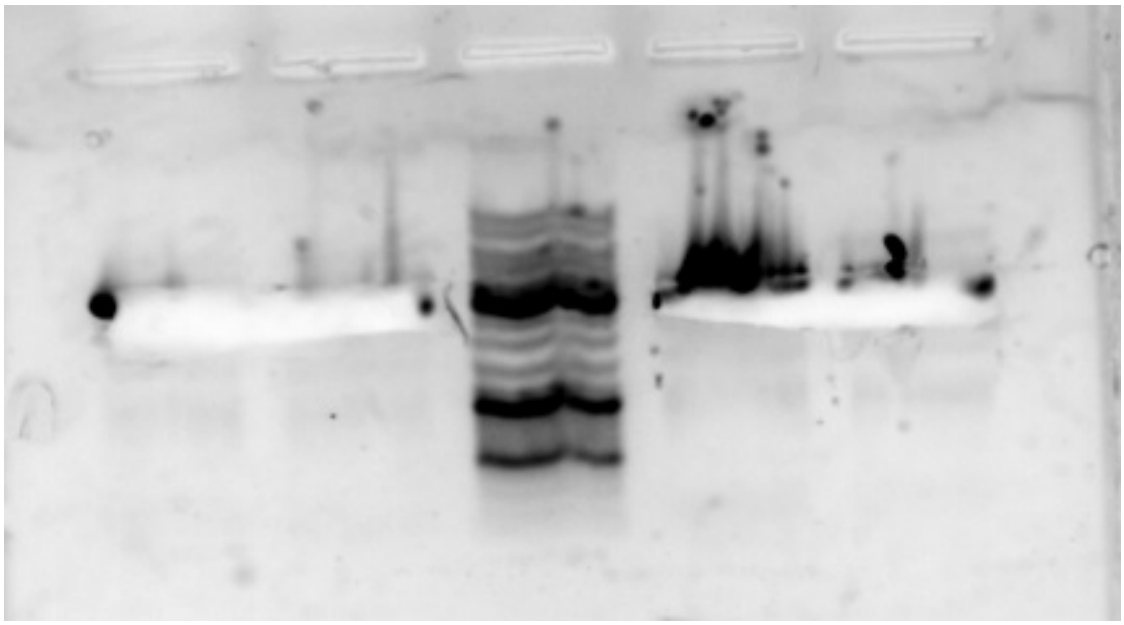


The excised bands were purified using GeneJet Gel Extraction kit (ThermoFisher) and the attached protocol. Elution was carried out to a volume of 30 uL, and DNA concentrations were determined using Nanodrop.

Table3

	A	B
1	Product	Concentration
2	QDR2I_Nstrep-C1	38.8 ng/uL
3	QDR2I_Cstrep-C2	37.8 ng/uL

TUESDAY, 8/2

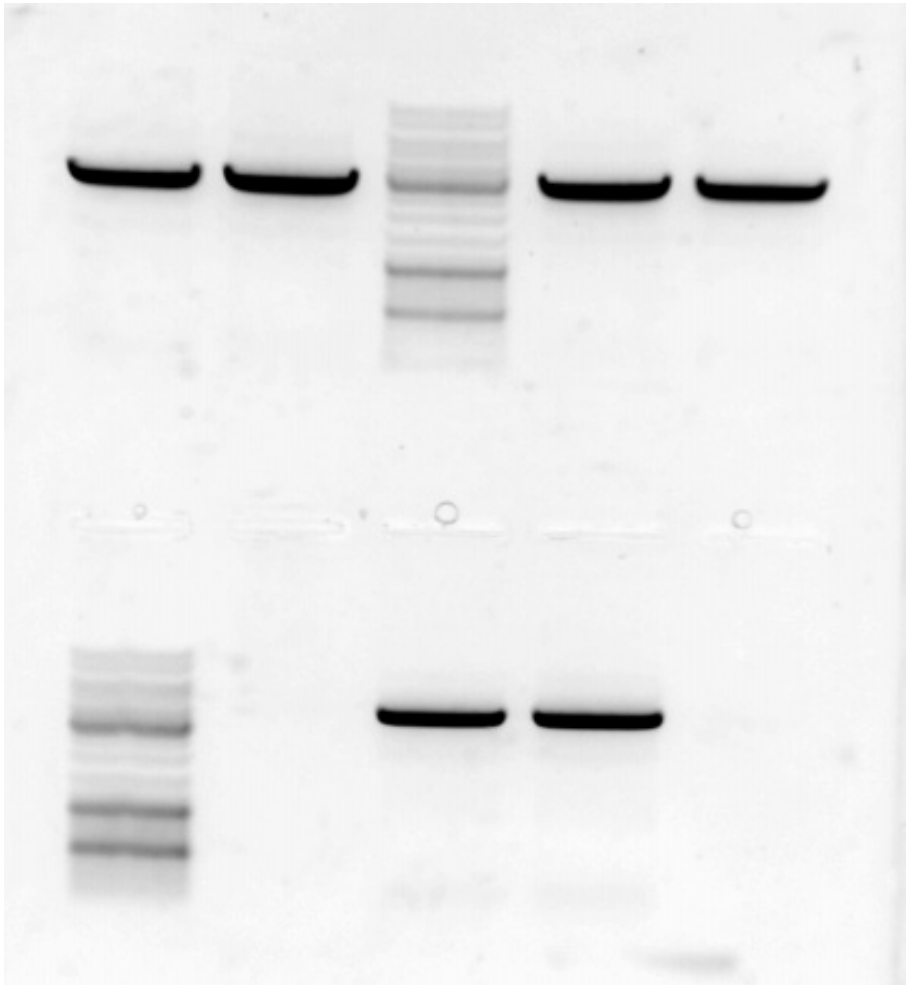
PCR was conducted for the remaining three transporter colonies using plasmid DNA purified from colonies. DNA was used from colonies pAH06-C2 (QDR2I), pAH07-C1 (QDR2L) and pAH10-C1 (QDR2I_mCherry). PCR reactions were set up as follows;

Table4

	A	B	C	D
1		pAH06-C2	pAH07-C1	pAH10-C1
2	Phusion Hotstart II Hifi Mastermix (2X)	50 uL	50 uL	50 uL
3	FW primer (PS3, 10 uM solution)	1 uL	1 uL	1 uL
4	RV primer (PS4, 10 uM solution)	1 uL	1 uL	1 uL
5	Template plasmid	0.4 uL (111 ng/uL solution)	0.7 uL (70 ng/uL solution)	0.8 uL (66.0 ng/uL solution)
6	H2O	47.6 uL	47.3 uL	47.2 uL
7	total	100 uL	100 uL	100 uL

Cycling conditions, and gel electrophoresis were conducted as previously. The gel with the samples was imaged;

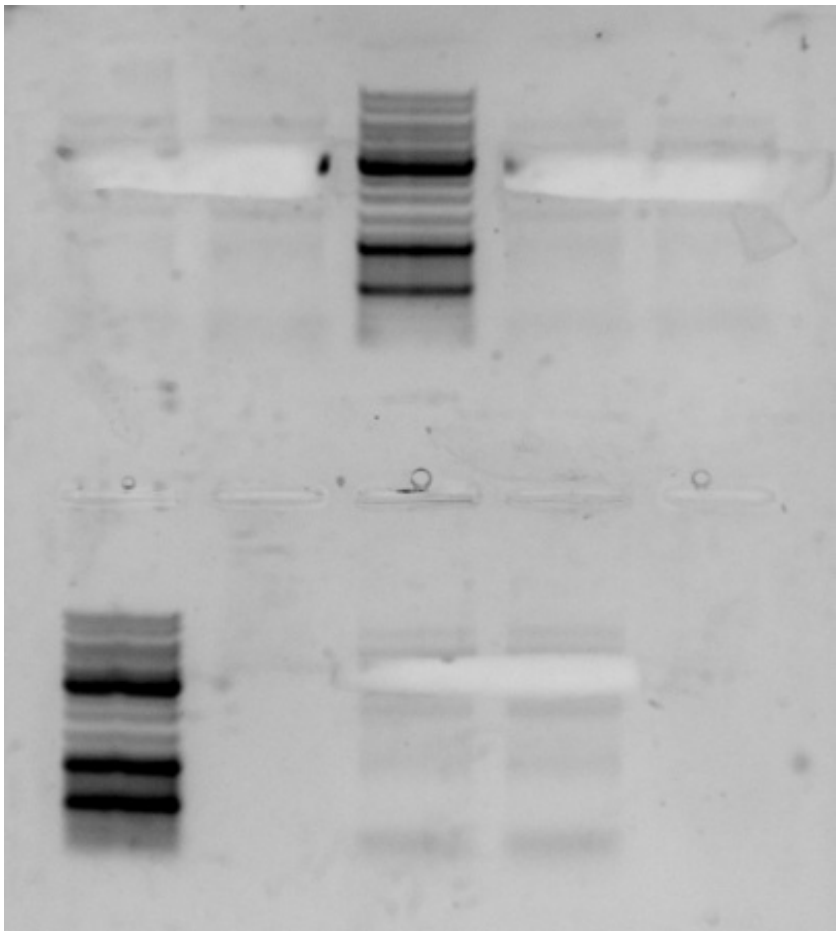
clipboard_2016-08-03_09:09:45.png



(from top left to bottom right; pAH6-C2 in 2 wells, marker, pAH07-C1 in 2 wells, marker, pAH10-C1 in 2 wells)

The correct bands appear around 3000 bp for pAH06 and pAH07, and uner 4000 bp for pAH10. The bands were cut out, and the gel was imaged again.

clipboard_2016-08-03_09:12:43.png



(from top left to bottom right; pAH6-C2 in 2 wells, marker, pAH07-C1 in 2 wells, marker, pAH10-C1 in 2 wells)

The excised bands were purified using GeneJet Gel Extraction kit (ThermoFisher) and the kit's protocol. Elution was carried out to a volume of 30 uL, and DNA concentrations were determined using Nanodrop.

Table5

	A	B
1	Product	Concentration
2	pAH06-C2	59 ng/uL
3	pAH07-C1	41.8 ng/uL
4	pAH10-C1	37.3 ng/uL

08-02 Genome Integration Transformation into W303alpha + Colony PCR

Project: Transporter
Authors: Pihla Savola
Dates: 2016-08-02 to 2016-08-08

TUESDAY, 8/2

Competent yeast cells were prepared from W303alpha strain according to Competent yeast cells -protocol. They were resuspended into 100 ul or 200 ul of solution A and stored in +4°C O/N.

Plates (YPD+G418) for plating transporter transformations were prepared. G418 stock 200 mg/ml in water was prepared. Working concentration of
G418 was 200 mg/l, so 0.4 ml of stock was added to 400 ml of YPD media.

WEDNESDAY, 8/3

Transformation was done according the transformation protocol, except now after heat shock cells were resuspended in 1 ml of YPD instead of 100 ul of water and incubated 3,5h at 30°C before plating them on YPD+G418 plates. Suggested DNA quantity for transformation was 500 ng, but at this case up to 1200 ng was added to reaction.

Following plasmids were transformed into W303a-leu.
-pAH06-C2
-pAH07-C1
-pAH08-C1
-pAH09-C2
-pAH10-C1

The concentration of plasmids was about 40 or 60 ng/ul so 30 or 20ul of these solutions was added respectively into transformation reaction.

FRIDAY, 8/5

Each transformation had succeeded and there were many colonies on each plate. Also some (n.20) colonies had grown on negative control plate.

Colony PCR was done from three colonies on each of the 5 plates (all together 15 colonies). Two primer sets were used for each colony: one that tells the precence of correct insert and the other one that tells if the new insert is missing, and the original gene is still there. Primer pair PS9+OJR95 should produce a PCR product when the transformation of QDR2 transporter has succeeded. Primer pair PS9+PS10 should produce a PCR product if our transformation has failed and original transporter of W303a has remained in the genome. Lenghts of these PRC product are 750bps and 270bps respectively.

PCR mastermixes was set according to the Colony PCR -protocol.

10 uL of each the mastermix was aliquotted into PCR tubes. Colonies were picked from pAH06-10 transformations, and additionally one reaction pair was set up using W303alpha as template, to be a negative control. THe picked colonies were

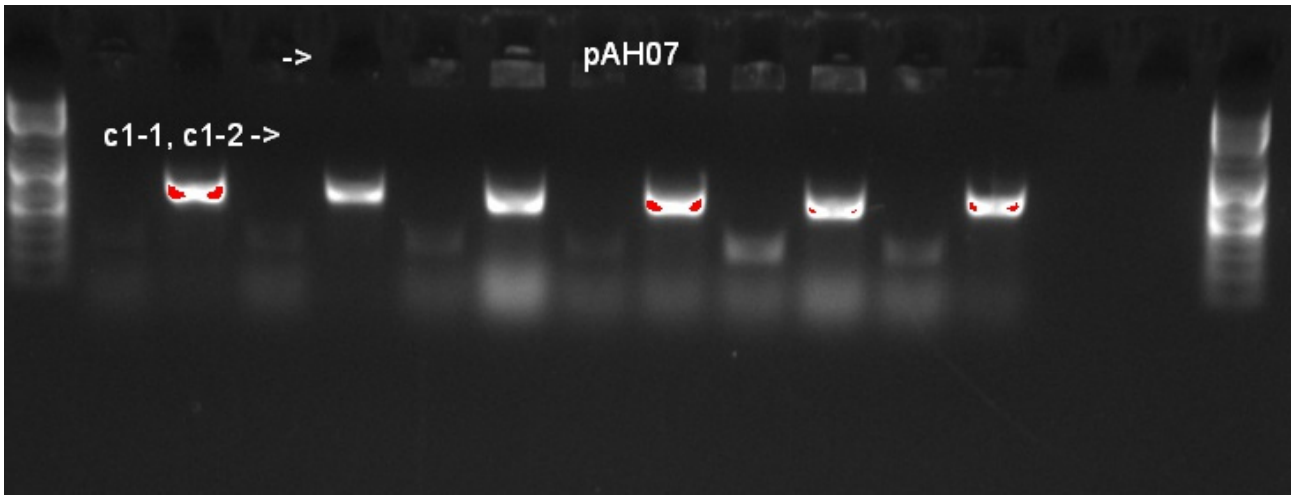
PCR cycling conditions were as follows;

Table1

	A	B	C
1	95 C	3 min	
2	95 C	30 s	
3	50 C	30 s	
4	72 C	50 s	cycle back to 2, x30
5	72 C	15 min	
6	4 C	hold	

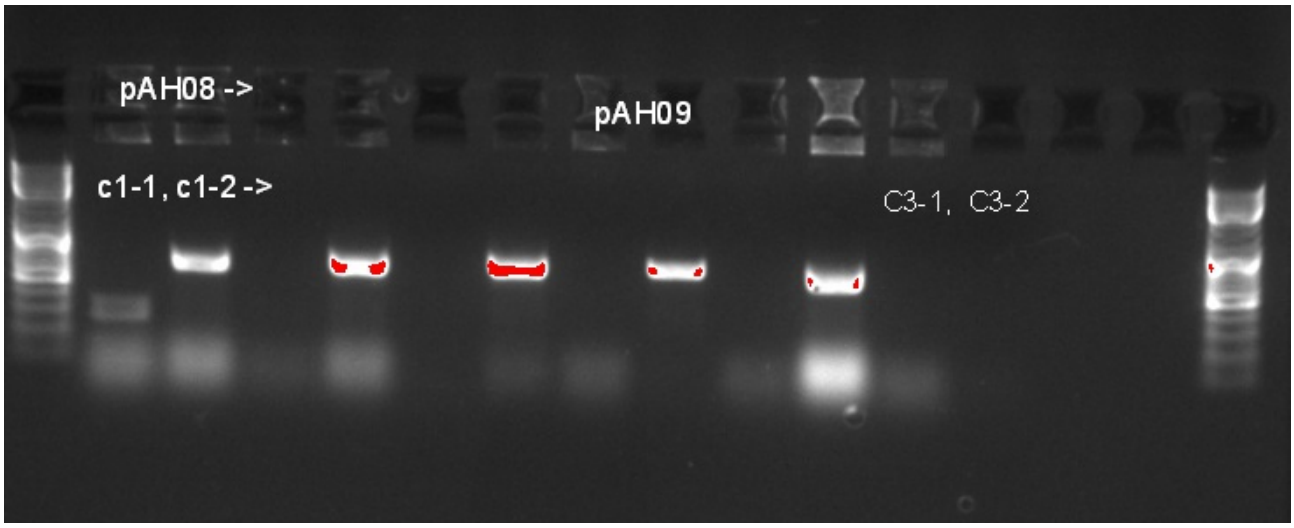
5 uL of PCR products were loaded on a 2 % agarose gel. The following images were taken after gel electrophoresis>

clipboard_2016-08-05_16:20:09.png



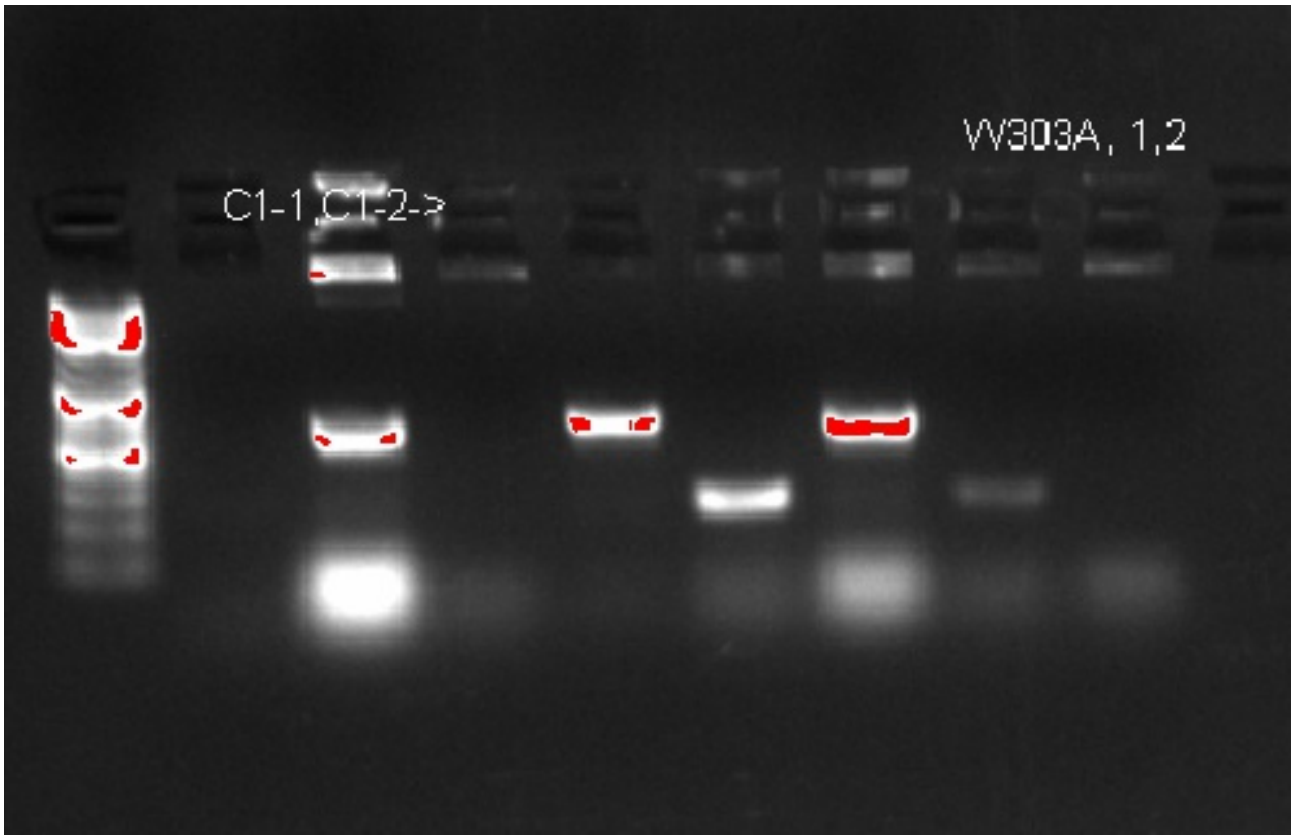
(from left; marker, pAH06-C2 colonies 1-3 (c1-1 means PS9+PS10 product, c1-2 means PS9+OJR95 product), pAH07-C1 colonies 1-3, 2 empty wells, marker)

clipboard_2016-08-05_16:20:23.png



(from left; marker, pAH08-C1 colonies 1-3 (c1-1 means PS9+PS10 product, c1-2 means PS9+OJR95 product), pAH09-C2 colonies 1-3, 2 empty wells, marker)

clipboard_2016-08-05_16:19:42.png



(from left; marker, pAH10-C1 colonies 1-3 (c1-1 means PS9+PS10 product, c1-2 means PS9+OJR95 product), W303alpha PCR products as negative control)

For pAH06 and pAH07, the band corresponding to the correctly integrated insert appears for all the colonies (around 700bp) when using primers to detect the integrated insert, but for all of the colonies, when using primers to detect the original gene, a band appears around 300bp, which would appear to correspond to new insert.

This same thing is observed for some of the other colonies (pAH08-C1 and pAH10-C3). Colonies pAH08-C2+3, pAH09-C1+2 and pAH10-C1+2 appear to produce the expected result for a correct insert; no product for primers targeting original insert and a 700bp product for the new insert.
Comparing to the W303alpha control, the 300 bp band clearly seems to correspond to the original, unmodified gene. However, since faint bands of this size are also observed in all pAH10 colonies when using primers to target the new gene, it is possible that this band would be an unspecific product. Because some of the picked colonies were quite large, it is also possible that what appeared to be one colony was in fact two, leading to an apparent dual result of new gene and old gene.

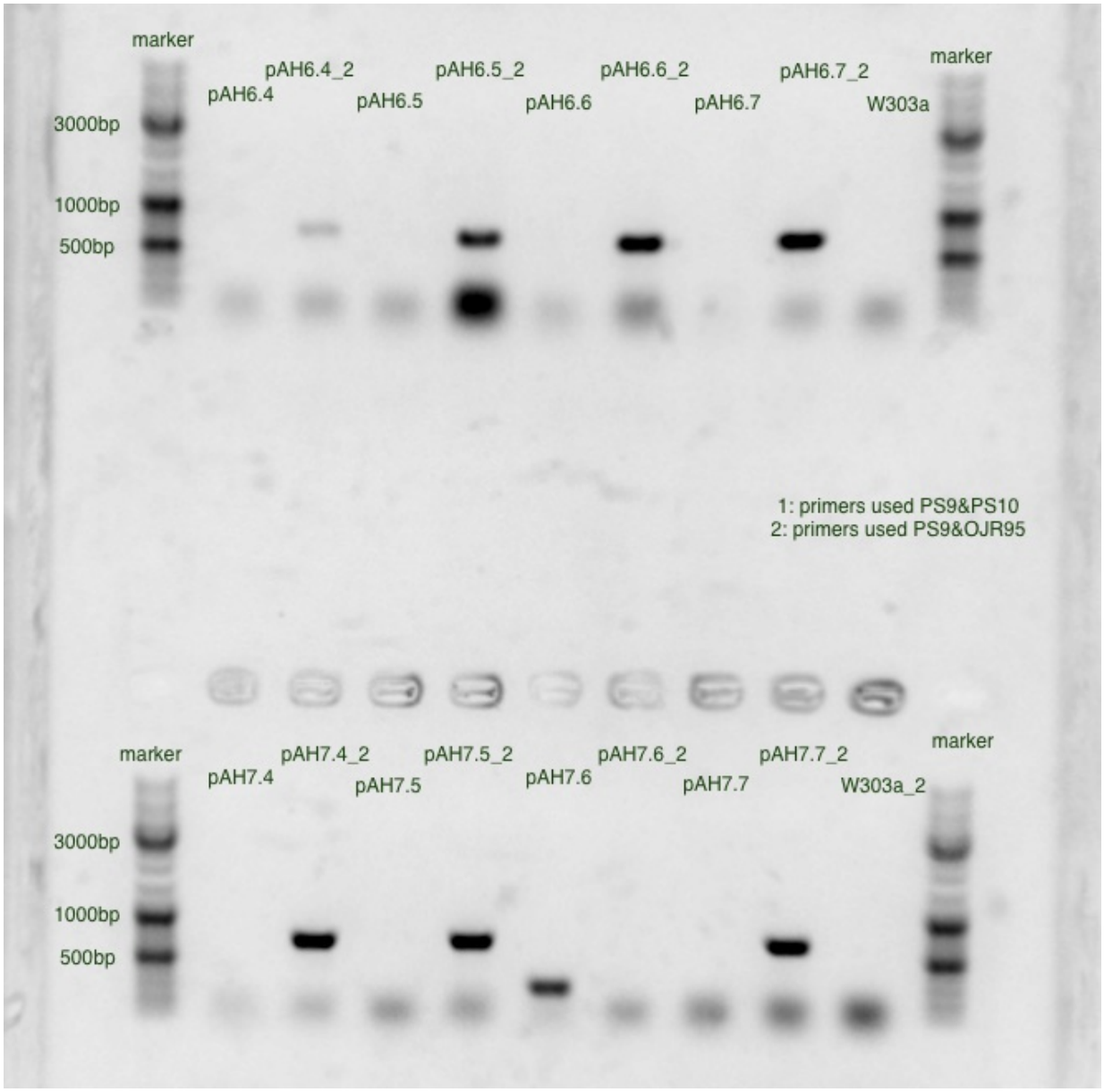
To test this hypothesis, colony PCR will be repeated for the colonies showing dual results, using single colonies from a plate freshly restreaked from the original colony. Addtionally, new colonies from the original plates can be tested for pAH06 and pAH07 to try and obtain a correct product.

For continued work, pAH08-C3, pAH09-C1, and pAH10-C2 were chosen.

MONDAY, 8/8

2 colonies of pAH6-C2 and pAH7-C1 from the restreaked plate and two from original plate were analysed with colony PCR. According the result each of the analysed colonies, exept pAH7.6 should have the correct insert. Colony PCR was done also from W303a for control. A band of size 200bp should be seen with primer set 1 (PS9&PS10), but in this case there is no band. This might be due to "pipetting" error. It can be that sample from w303a was added into tube 2 twice, so that no cells would have been added in to tube one explaining the absence of the band. Anyhow as pAH7.6 clearly explains what no insert sample looks like, we can be sure that other samples had the correct insert in them.

Näyttökuva 2016-08-08 kello 15.39.26.png



08-08 Transporter assay with H2O2 in W303alpha

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-08-08 to 2016-08-09

MONDAY, 8/8

Liquid cultuces from each W303a, VL3, pAH6-C2-C7, pAH7-C1-C4 and pAH10-C1-C2 were prepared in YPD (without antibiotic!).

TUESDAY, 8/9

There were no cells in the pAH6 culture, but since pAH10 (construct with transporter fused to mCherry) had also I as the amino acid in I/L location, that could be used instead of pAH6.

Cells were centrifuged 5000rpm/5min and diluted into 5ml 2X PBS. OD600s were measured from each sample and they were diluted to reach the same OD600 in each sample.

Table1

	A	B	C	D	E	F
1		OD600	1:100	real OD	dilution made	OD(after dilution)
2	W303	2,94	0,326	32,6	1:5	6,5
3	VL3	3,047	0,46	46,0	1:6	7,6
4	pAH7-C1-C4	2,846	0,249	24,9	1:5	5,0
5	pAH10-C1-C2	2,957	0,298	29,8	1:5	6,0

Solutions of microcystin were made from methanol extract containing 50 ug/mL. Solutions were prepared to be 2X of the desired sample concentration, as final samples are made with 1 : 1 cell suspension and toxin solution.

Table2

	A	B
1	Final intended MC concentration in samples (ug/L)	Prepared MC solution (ug/L)
2	0	0
3	1	2
4	10	20
5	100	200
6	1000	2000

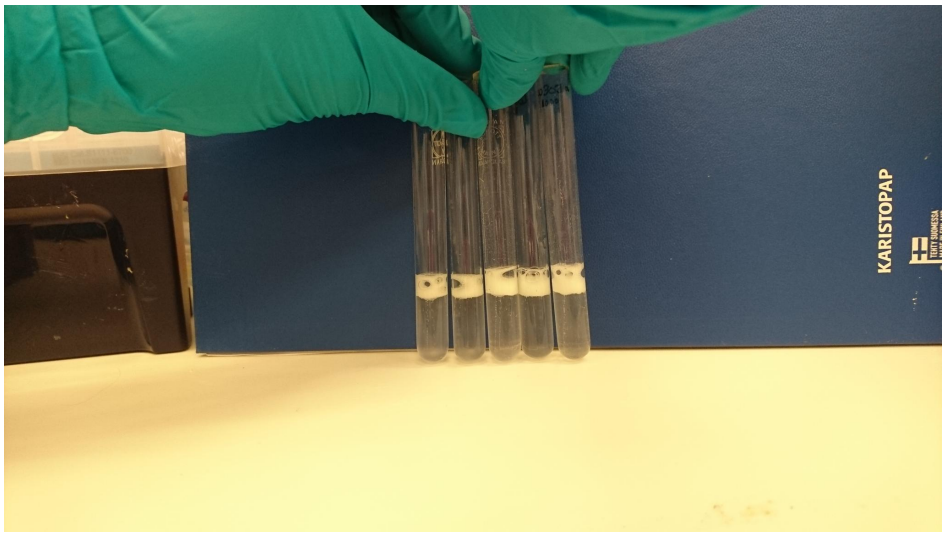
Solutions were prepared by mixing 0.6 mL of the stock MC extract (50 ug/mL) with 14.4 mL of water to prepare 2000 ug/L solution. Serial dilutions of 200, 20, and 2 ug/L were made by combining 1.5 mL of the previous solution with 13.5 mL of water.

Transporters were tested according to the attached protocol with H2O2. 0,5 ml of cell solution and 0,5 ml of 2X MC solution (concentrations presented in table 2) was pipetted into glass tubes and incubated in RT for 1h. 4 different toxin concentrations were tested as well as the sample without any toxin. After the incubation, 0,5 ml of H2O2 and 0,2 ml of TritonX-100 were pipetted into each tube. After 5 minutes picture was taken from tubes.

NOTE! In toxin tests from Viikki there was no MC in our MC extract meaning that toxin concentration in each solution was 0. Test must be repeated with new extracts. Additionally, all handling of MC must be done with glassware, including using glass pipettes, as MC sticks to plastic.

Pictures from foam layers: W303, VL3, pAH07, pAH10

W303alpha 0-1000.JPG

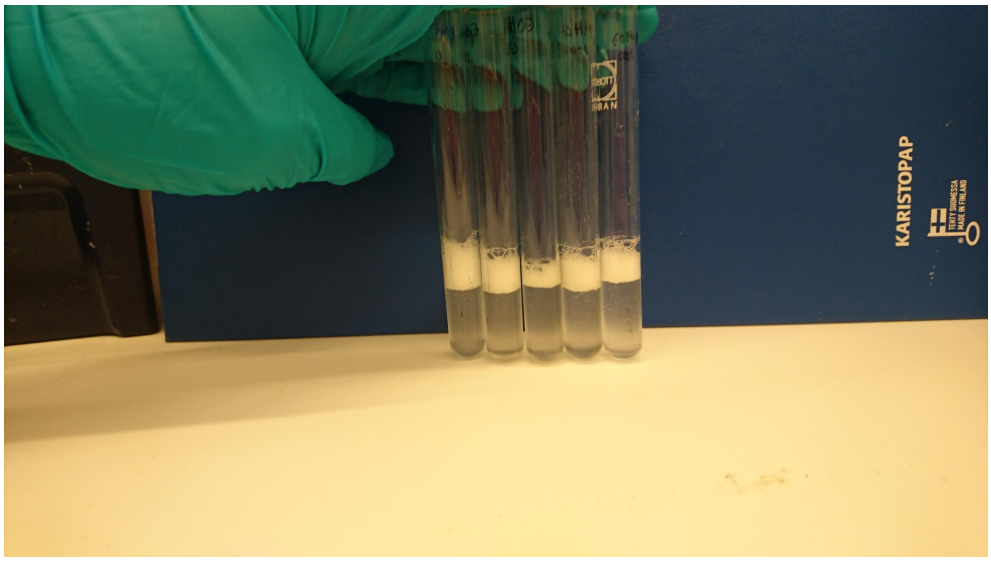


From left; samples solutions with supposed MC concentration of 0, 1, 10, 100 and 1000 ug/L, in W303alpha strain.

VL3 0-1000.JPG

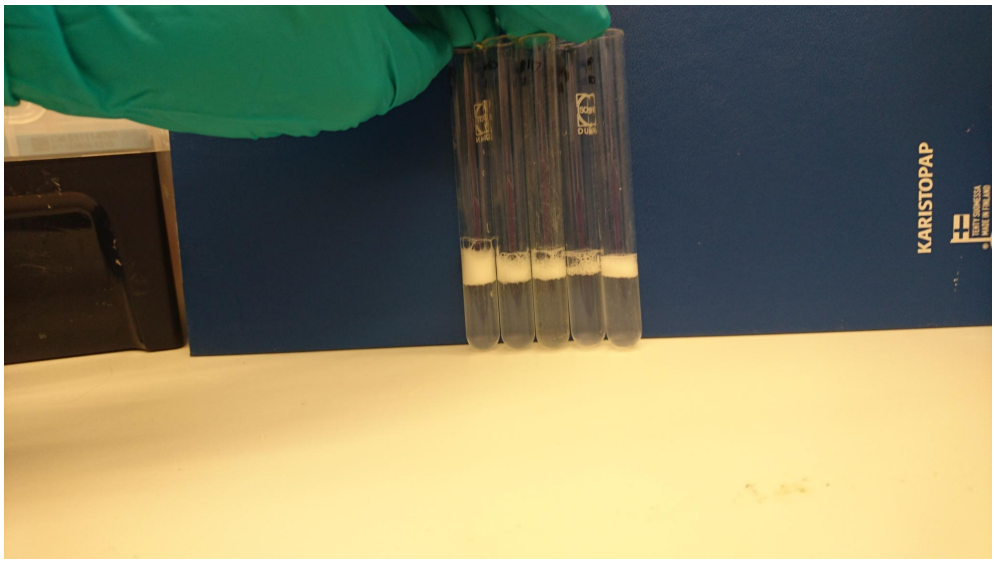
From left; samples solutions with supposed MC concentration of 0, 1, 10, 100 and 1000 ug/L, in VL3 strain.

pAH07 2.JPG



From left; samples solutions with supposed MC concentration of 0, 1, 10, 100 and 1000 ug/L, in W303alpha strain with pAH07 transporter.

pAH10 0-1000 2.JPG



From left; samples solutions with supposed MC concentration of 0, 1, 10, 100 and 1000 ug/L, in W303alpha strain with pAH10 transporter.

As can be seen, foam layer in VL3 is much thicker than in other samples. pAH07 produces more foam compared to W303a. pAH10 produces about same amount of foam as W303a. Since there was no microcystin in the toxin solutions this experiment just tells about the normal stress response of yeast cells. W303a strain has a certain deletion that affects the accumulation of oxidative stress related transcription factors in nucleus and this might be the reason why the stress response to H2O2 is significantly lower than in VL3. Anyway, changing the transporter affects the thickness of foam layer, meaning that it must be somehow associated to stress reaction. So does the transport transport also something else?

Further experiments will be necessary with a different yeast strain, proper MC extracts and handling MC solutions correctly with only glasssware.

08-11/26 Retransformation into SS328-leu + colony PCR screening

Project: Transporter
Authors: Pihla Savola
Dates: 2016-08-11 to 2016-09-07

THURSDAY, 8/11

To prepare cells for transformation, *S. cerevisiae* strain SS328-leu (SS328 strain (https://www.lgcstandards-atcc.org/products/all/MYA-193.aspx?geo_country=fi) with additional deletion for leucine auxotrophy. This deletion has been selected for using nourseothricin) was streaked onto YPD plates from a glycerol stock and grown for 1 day at 30 C and 3 more days in room temperature.

MONDAY, 8/15

Liquid cultures (3x5ml) from SS328-leu were prepared in YPD. SS328 was also restreaked onto a new plate.

TUESDAY, 8/16

Dilutions from liquid culture were made to reach a starting OD of 0,5. Incubation in 30°C was continued.
Competent cells were done according the protocol and suspended in 200 ul of solution A. 100 ul was used for one transformation reaction.

Following constructs were transformed in SS328-leu according the LiAC knockout transformation:

-pAH6-C1
-pAH6-C2
-pAH7-C1
-pAH8-C1
-pAH8-C3
-pAH8-C5
-pAH10-C1
-pAH10-C2

200 ul of each transformation reaction was plated in YPD + G418 + 1xNAT plates and in YPD + G418 + 2xNAT

THURSDAY, 8/18

Many colonies resulted in plates with both NAT concentration. There were no clear single colonies so next time plating less than 200 ul would be a good idea. Now a colony from following plates wes restreaked onto a new YPD +G418 +2xNAT plates:

-pAH6-C1
-pAH6-C2
-pAH7-C1
-pAH9-C2
-pAH10-C1
-pAH10-C2

Plates were incubated one day at 30 C and two more at room temperature.

SUNDAY, 8/21

Growth on restreaked plates was poor, with few colonies, and hardly any well separated single colonies. To facilitate screening, single colonies were inoculated into culture tubes with 5 mL of YPD medium and grown overnight at 30 C, 220 rpm shaking. Some of the colonies were from the original transformation plates, although the colonies were small and possibly mixed, as only one colony had been streaked on the new plates.

MONDAY, 8/22

Colony PCR was set up according to the attached protocol to screen from the liquid samples. A total of 14 colonies were chosen; 4 of pAH06-C2, 3 of pAH07-C1, 3 of pAH09-C2 and 4 of pAH10-C1. For each colony, two PCR reactions were needed ("+" to verify correct insert, and "-" to verify unmodified gene.)

Master mixes were set up as follows;

Table1

	A	B	C
1		+	-
2	DreamTaq Green Master Mix	80 uL	80 uL
3	PS9 (FW primer)	3.2 uL	3.2 uL
4	PS10 (RV primer)		3.2 uL
5	OJR95 (RV primer)	3.2 uL	-
6	H20	70.4 uL	70.4 uL

9.8 uL of these mastermixes were aliquotted into PCR tubes, and 0.2 uL of template culture was added (from each culture, both + and - reactions were prepared.) Cycling conditions were as follows:

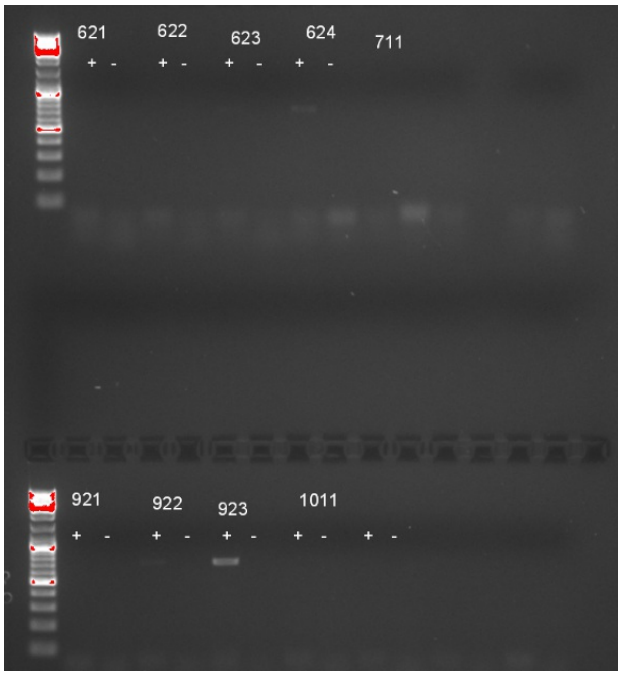
Table3

	A	B	C
1	95 C	3 min	
2	95 C	30 s	
3	50 C	30 s	
4	72 C	50 s	cycle back to 2, x30
5	72 C	15 min	
6	4 C	hold	

Primer pair PS9+OJR95 should produce a PCR product when the transformation of QDR2 transporter has succeeded. Primer pair PS9+PS10 should produce a PCR product if our transformation has failed and original transporter of W303a has remained in the genome. Lenghts of these PRC product are 750bps and 270bps respectively.

7 uL samples of each PCR was run on a 2 % agarose gel. The Imaged gel is attached below.

clipboard_2016-08-25_14:58:05.png



(from top left; pAH02-C2-C1, pAHC2-C2 etc. until pAH-C1-C3 and bottom left; pAH09-C2-C1, etc. until pAH10-C1-C4)

Although it seems like some correct products can be seen (pAH06-C2-C4 and pAH09-C2-C3), since the PCRs in general seem to have not worked and not produced bands, it seems possible that even these samples could have e.g. been mixed colonies, but the - band to indicate this hasn't appeared because of PCR failure. For this reason, it was chosen to restreak multiple colonies from the original transformation plates and screen these. Restreaking was done for the following constructs, streaking 3-4 colonies per plate and doing 2X and 1X NAT plates for each construct:

-pAH06-C1
-pAH06-C2
-pAH07-C1
-pAH09-C2
-pAH10-C1
-pAH10-C2

WEDNESDAY, 8/24

Single colonies were observed on restreaked plates, but in some cases scolonies were still tightly spaced together. Colony PCR was conducted from colonies according to the attached protocol, with the principle of doing + and - reactions of each colony. Two colonies were picked from each of the restreaked constructs (above). In addition, reactions were prepared for a colony of SS328-leu to make a negative control, for a total of 13 colonies (26 reactions).

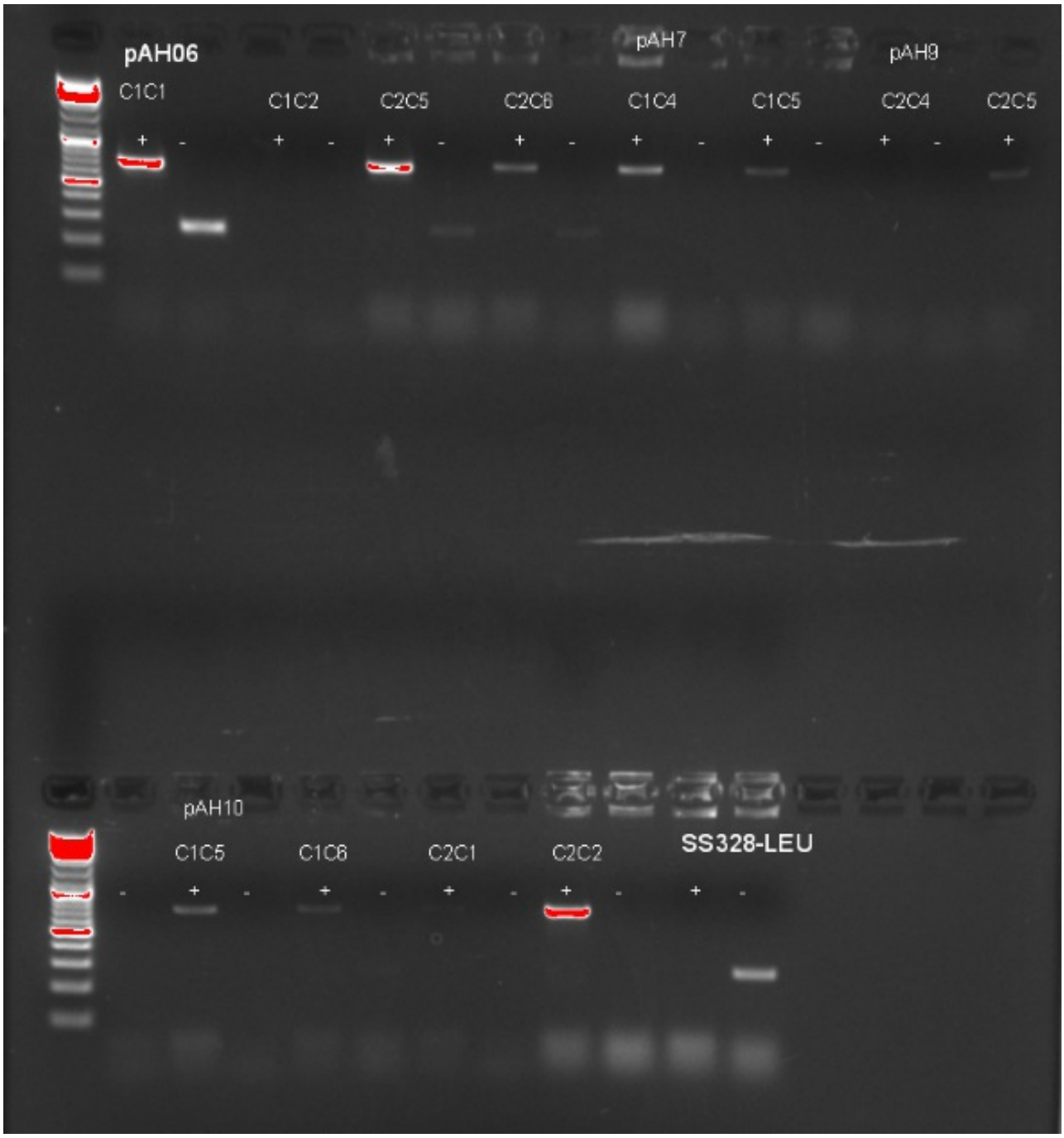
Mastermixes were set up as follows

Table2

	A	B	C
1		+	-
2	DreamTaq Green Master Mix	80 uL	80 uL
3	PS9 (FW primer)	3.2 uL	3.2 uL
4	PS10 (RV primer)		3.2 uL
5	OJR95 (RV primer)	3.2 uL	-
6	H20	73.6 uL	73.6 uL
7		160 uL	160 uL

10 uL reactions were aliquotted. The leftover mastermixes were stored in -20 C. Reactions were inoculated with the proper colonies using sterile pipette tips and PCR was conducted in the same cycling conditions as before. 5 uL of products were run on a 2 % agarose gel as before and the visualization is as follows:

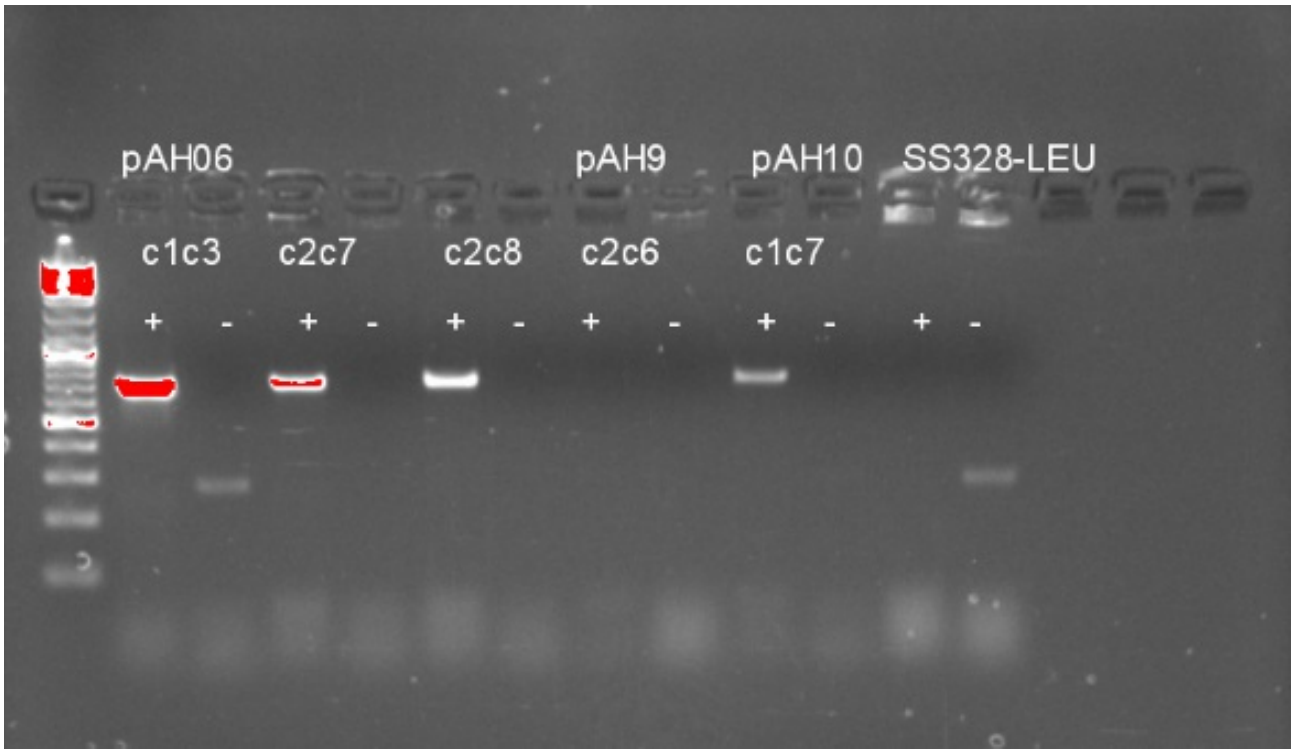
clipboard_2016-08-25_15:56:01.png



Based on the image, pAH07-c1-c4, pAH07-C1-C5, pAH09-C2-C5, pAH10-C1-C5, pAH10-C2-C1 and pAH-C2-C2 contain the correct insert (pAH10-C2-C2 band appears very faint here but it can be seen). The other colobues appear to have been mixed colonies, or no products have been formed. No products being formed could be due to very small colonies, of which obtaining two colony PCR samples is difficult.

Some additional colonies were screened; the procedure was before (with the exception of mastermix total volumes) and the result was as follows:

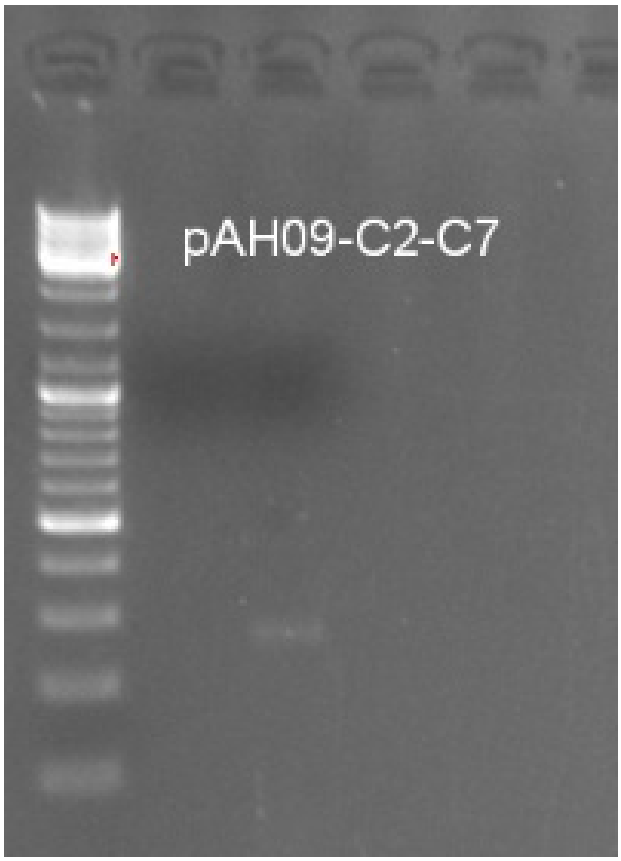
clipboard_2016-08-25_16:04:26.png



Based on this image, pAH06-C2-C7, pAH06-C2-C8 and pAH10-C1-C7 seem to be the correct; the others are mixed or no product is formed. Because obtaining single colonies from pAH06-C1-C3 and pAH09-C2-C6 seemed difficult, colonies from their restreaked plates were restreaked once more, still on the selective plates.

One more colony was screeened from pAH09-C2 in the hopes of obtaining 2 correct clones for each construct. 10 uL of the frozen leftover mastermix from the day's first PCR was used for this. The result is as follows: this colony contained the original gene.

clipboard_2016-08-25_16:08:53.png



Overnight liquid cultures were then prepared from all of the screened colonies that contained the new insert. For each culture, 5 mL of YPD medium in a 50 mL falcon tube was inoculated with the remains of the colony. For some of the colonies, a small possibility exists that some mixed colony was inoculated, as the colonies grew so close together.

The cultures were grown at 30 C with shaking at 240 rpm, starting at 8pm

THURSDAY, 8/25

Because new sequencing results confirm the pAH06-C2 to be correct, work on pAH06-C1 was discontinued. pAH10-C1, on the other hand, contained a single deletion in the protein coding sequence, so pAH10-C2 was chosen instead for further work. Sequencing for pAH10-C2 should be done. pAH07-C1 and pAH09-C2 were confirmed to be correct already earlier.

Because of the slight possibility of mixed colonies in the prepared liquid cultures, further verification was decided on before continuing with experiments. In the morning, 20 uL of liquid cultures were plated on selective YPD plates (1xNat+G418). From here, single colonies should be observed, and additional colony PCR can then be conducted on properly isolated colonies before restreaking thse colonies for making final stocks and plates for experiments.

At 9am, The following colonies were replated:

- pAH06-C2-C7
- pAH06-C2-C8
- pAH07-C1-C4
- pAH07-C1-C5
- pAH10-C2-C2

Hardly any growth was visible on pAH09-C2-C5 and pAH10-C2-C1 (pAH09-C2-C5 OD 0.1), so incubation with shaking was continued. For pAH09-C2-C5, 1 mL was also inoculated in 4 mL of fresh YPD medium.

At 4pm, as growth still looked limited, cultures were pelleted and resuspended in 5 mL fresh YPD medium for incubation overnight, again at 30 C and 240 rpm shaking.

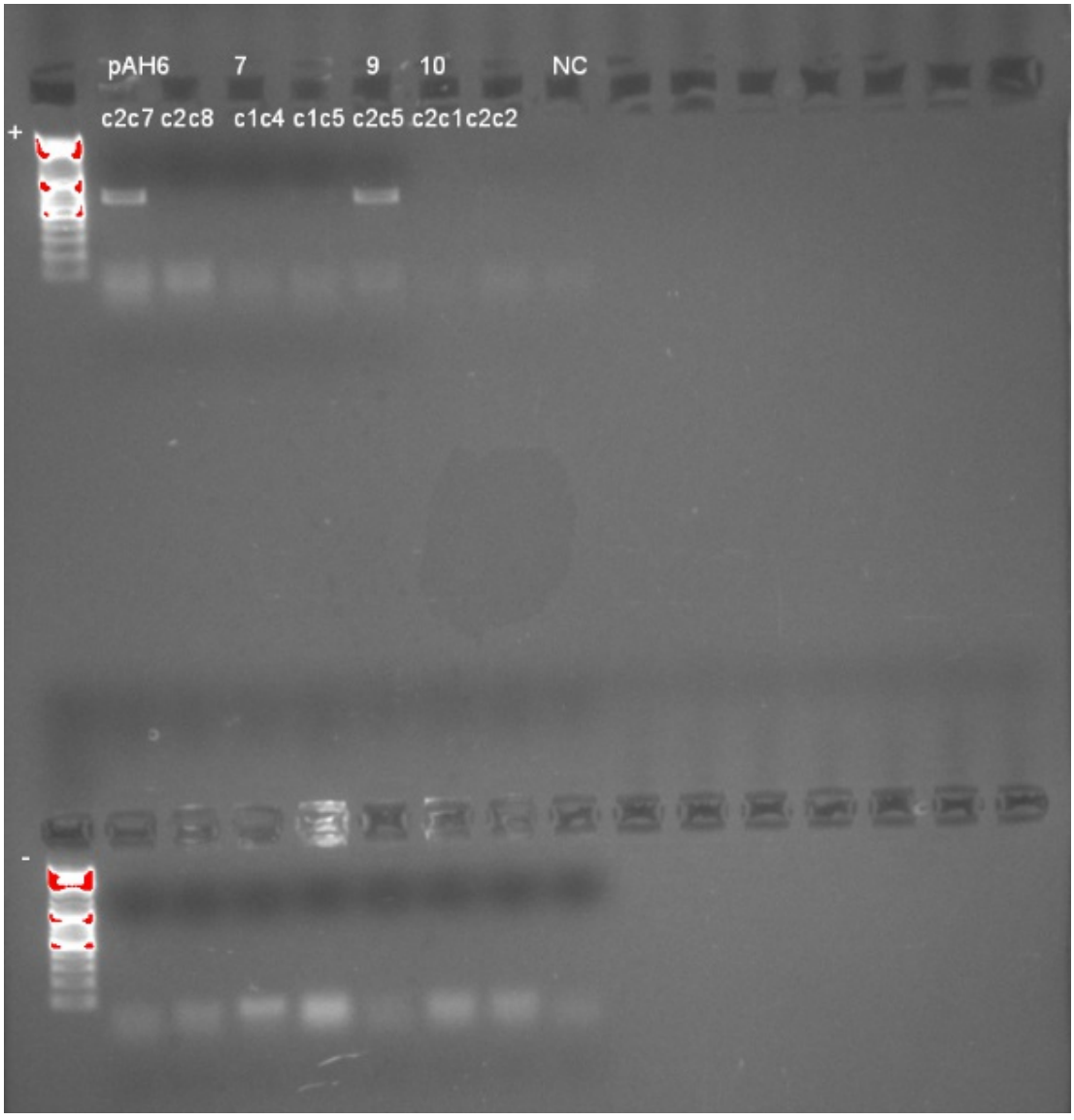
FRIDAY, 8/26

All cultures appeared cloudy in the morning, so 20 uL of both pAH09-C2-C5 and pAH10-C2-C1 was plated on selective Nat+G418 plates.

SUNDAY, 8/28

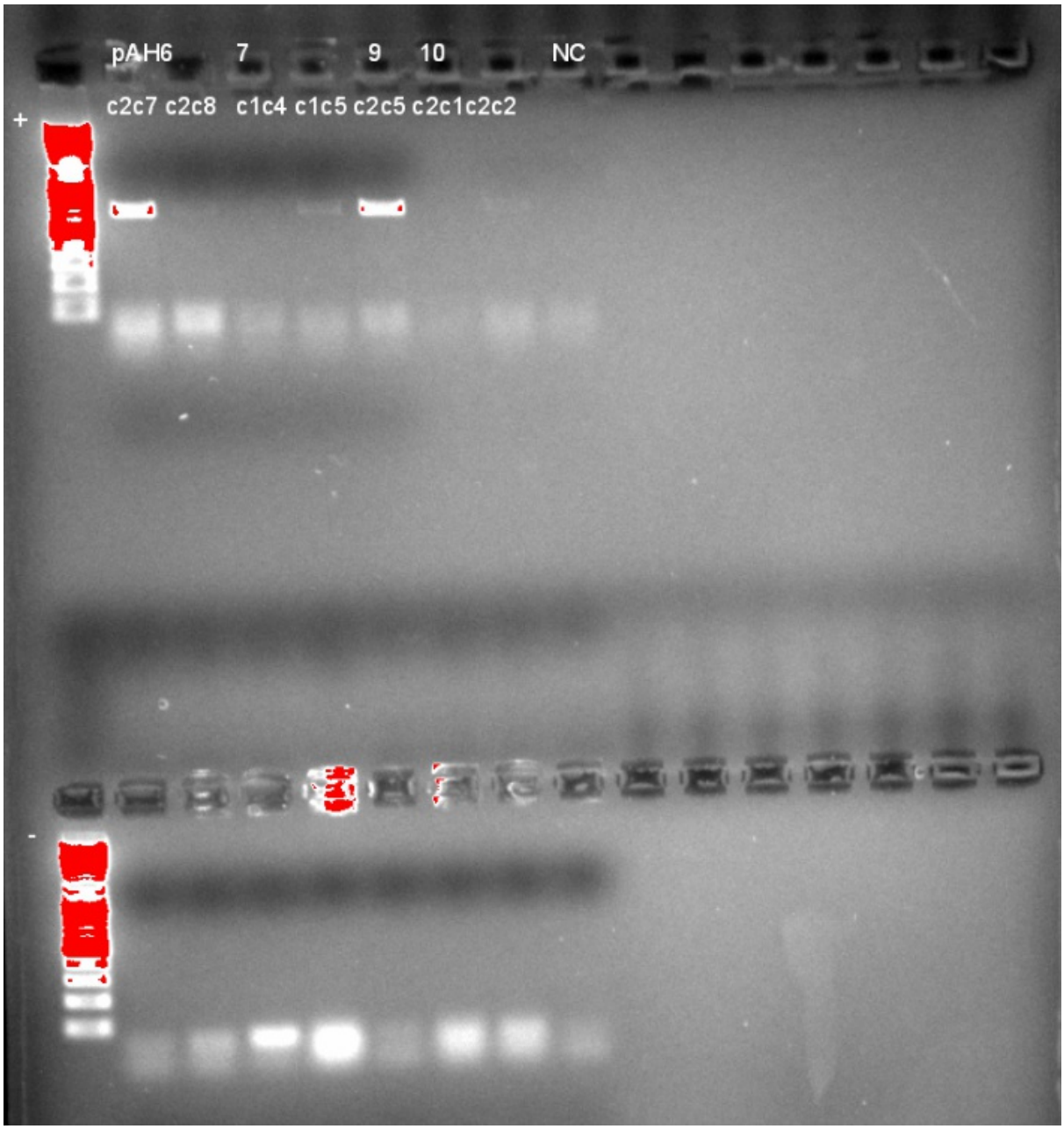
Single colonies were observed on the replated plates. For additional verification of the insert (as it is possible that these plates were made of mixed cultures), colony PCR was conducted as before on one colony from each plate. SS328-leu was used as a negative control)

clipboard_2016-08-29_11:35:30.png



(OJR95+PS9 products in top row; PS10+PS9 products in bottom row)

clipboard_2016-08-29_11:32:37.png



(OJR95+PS9 products in top row; PS10+PS9 products in bottom row)

The bands visible in the top row are the correct ones, although some of them are very faint, and only visible in the lower picture (using a protocol optimized for faint bands. What seems remarkable, however, is that no bands at all are observed for PS10+PS9 products, not even in the negative control. All colonies should produce a band for either PS9+PS10 or PS9+OJR95.
Because colonies were well isolated, however, the colonies showing up as positve here (pAH6-C2-C7/8, pAH07-C1-C5, pAH09-C2-C5 and pAH10-C2-C2) contain most likely only the correct, new transporter. Liquid cultures in YPD were prepared for these colonies from the remains of the colony on the plate and grown O/N at 30 c with shaking to prepare glycerol stocks and new, better verified plates.

As it is possible that variabilities in picked colony amounts could have caused some of the failed reactions, a new colony PCR was set up for second colonies from the same plates, this time in a different way:

- 50 uL of autoclaved water was aliquotted into sterile 200 uL eppendorfs
- Colonies were picked with a loop and swirled in the water
- 1 uL of this solution was used as template for the PCR reaction

Mastermixes for the PCR were set up as follows, for 9 PCR reactions (8 colonies, 1 negative control as before and 1 extra):

Table4

	A	B	C
1		+	-
2	DreamTaq Green Master Mix	45 uL	45 uL
3	PS9 (FW primer)	1.8 uL	1.8 uL
4	PS10 (RV primer)	-	1.8 uL
5	OJR95 (RV primer)	1.8 uL	-
6	H20	32.4 uL	32.4 uL
7		81 uL	81 uL

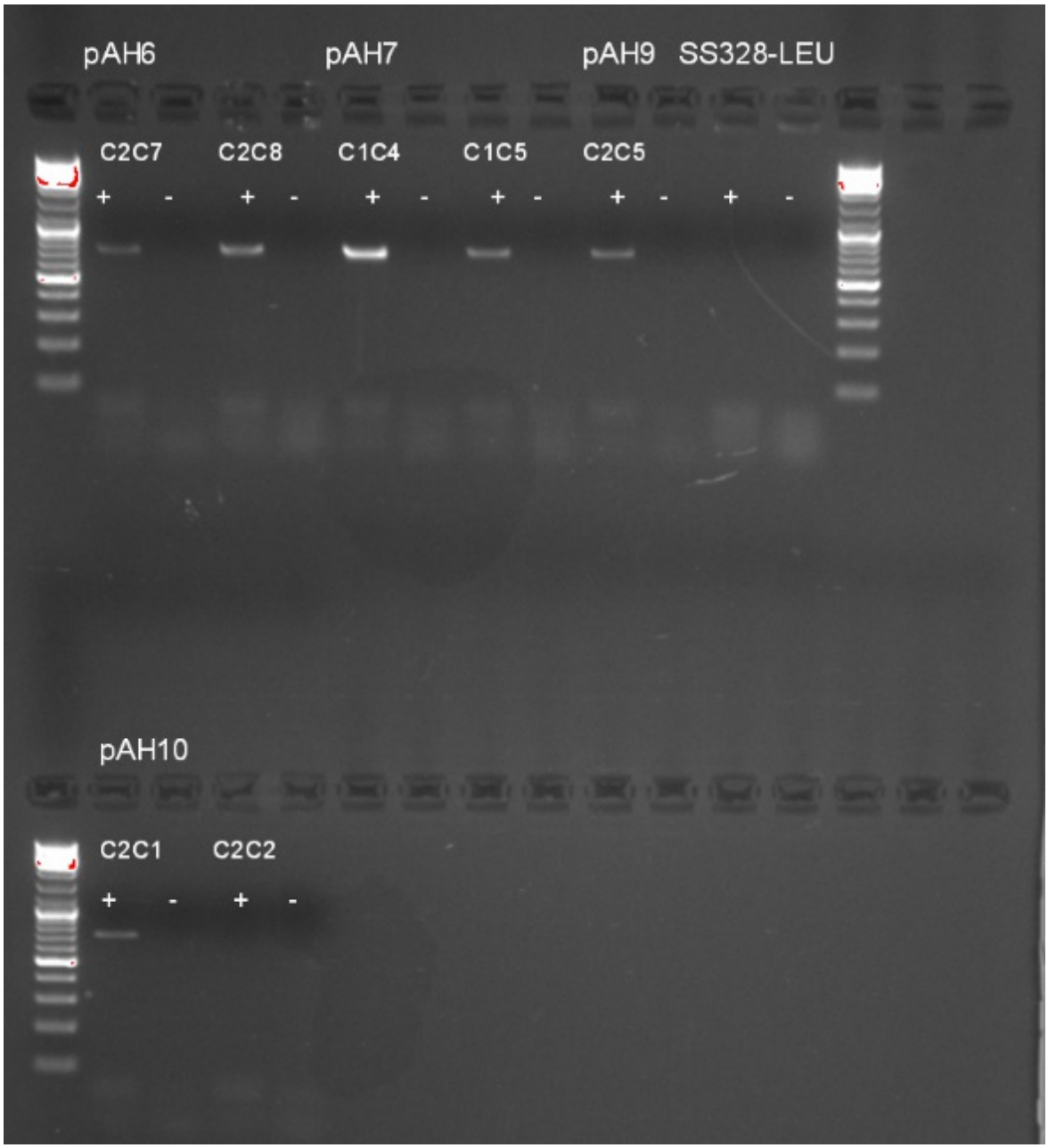
9 uL of the mastermixes was aliquotted into PCR tubes, and the1 uL template dissolved in water was added, after which PCR followed in the same cycling conditions as before.

To prepare alternative liquid cultures to the ones prepared from the plates, 30 uL of the template solution in water was inoculated in 5 mL of YPD and grown O/N in 30 C with shaking. The rest of the template solution was stored in +4 C.

MONDAY, 8/29

The results of the PCR using liquid template was visualized:

clipboard_2016-08-29_11:52:33.png



All except for pAH10-C2-C2 appear postive here (contain the new transporter), but again, no bands are visible for PS9+PS10, not even in the negative control; it would seem that PS10 might be degraded. The colonies showing the correct product were from well-isolated colonies that had once already been screened with a working negative control, however, so they most likely contain only the correct, new transporter.

All overnight cultures seemed to exhibit growth in the morning. The cultures prepared using water-based inculum were used for making glycerol stocks (500 uL medium + 500 uL 50% glycerol) and plates (20 uL of culture plated on YPD plates). The exception was pAH10-C2-C2, for which only the culture inoculated from a plate appeared to contain the correct insert.

MONDAY, 9/5

Colony PCR was done on colonies from verified transporters to check also the 5' integration site, in addition to the 3' site. A colony was suspended from each of the following plates in 50 uL of sterile water:

- pAH06-C2-C7
- pAH06-C2-C8

- pAH07-C1-C4
- pAH07-C1-C5
- pAH09-C2-C5
- pAH10-C2-C1
- pAH10-C2-C2
- SS328-leu (negative control)

3 PCR reactions were set up for each colony;

- One to produce a 447 bp product if the 5' integration site is correct
- One to produce a 701 bp product for the correct 3' integration site
- One to produce a 269 bp product if the 3' site contains the unmodified old transporter

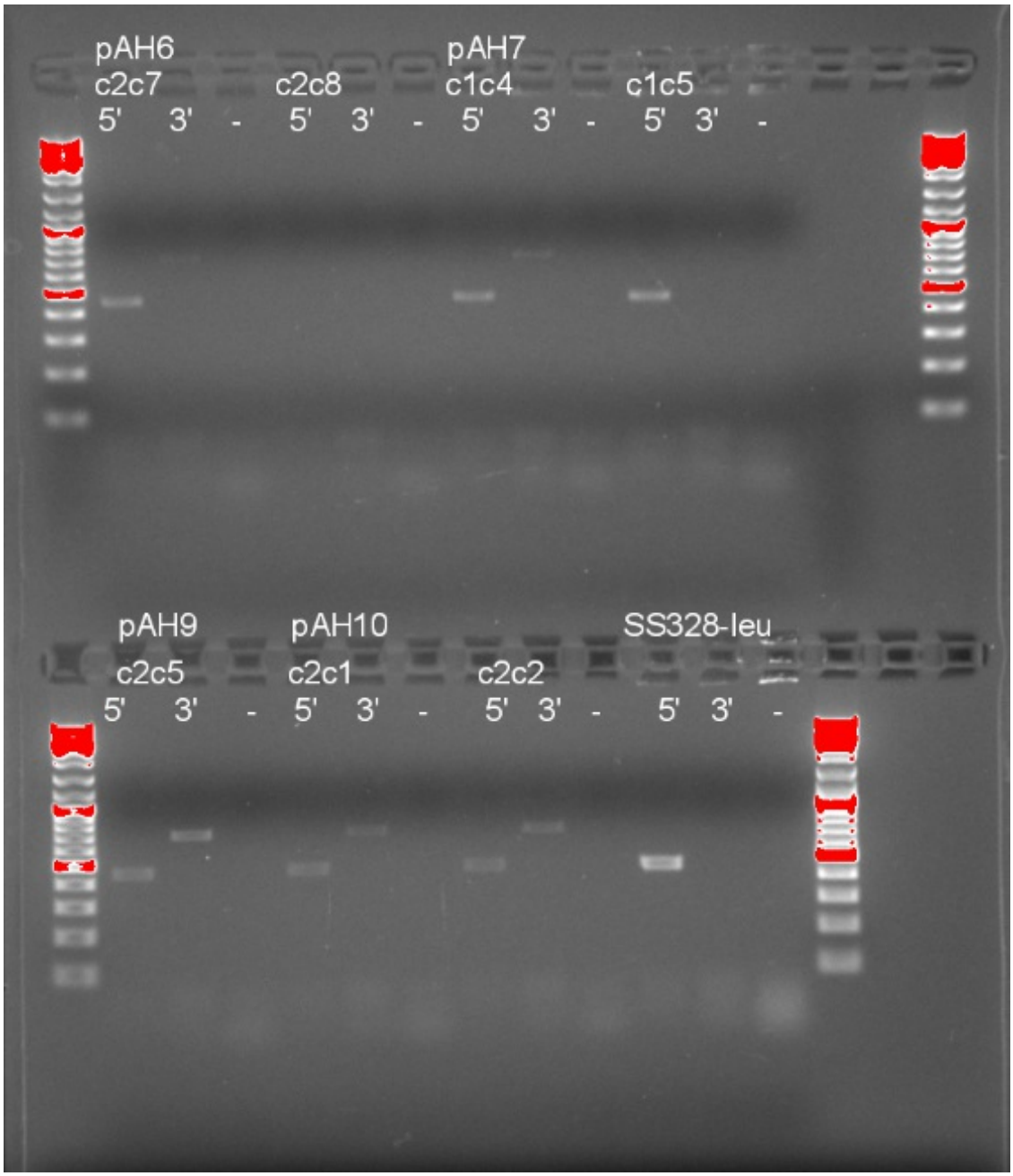
PCR mastermixes were set up as follows:

Table5

	A	B	C	D
1		5'	3'	-
2	DreamTaq Green Master Mix	45 uL	45 uL	45 uL
3	PS9 (FW primer)	-	1.8 uL	1.8 uL
4	PS10 (RV primer)	-	-	1.8 uL
5	PS11 (FW primer)	1.8 uL	-	-
6	PS12 (RV primer)	1.8 uL	-	-
7	OJR95 (RV primer)	-	1.8 uL	-
8	H20	32.4 uL	32.4 uL	32.4 uL
9		81 uL	81 uL	81 uL

9 uL of the mastermixes was aliquotted into tubes (8 reactions of each mastermix), and 1 uL of the template was added. PCR cycling protocol was as before. 5 uL of each reaction was run on a 2 % agarose gel (100 V, 400 mA, 20 min):

clipboard_2016-09-06_17:32:04.png



Based on this, the following constructs are correct in both integration sites:

- pAH06-C2-C7
- pAH07-C1-C4
- pAH09-C2-C5
- pAH10-C2-C1
- pAH10-C2-C2

pAH06-C2-C8 isn't correct, and pAH07-C1-C5 lacks the band for 5' end.

However, it is noted that the negative control produces a band for the 5' end, which it shouldn't, and no band for the negative control reaction, which it should. Although a fresh dilution of PS10 was used, it looks like the negative control reaction is working. The 5' band for SS328-leu is mysterious.

TUESDAY, 9/6

Strains containign replaced transporters were restreaked on G418-NAT plates. A negative control of just SS328-leu was also streaked on the plates.

WEDNESDAY, 9/7

All selective plates with the exception of the negative control and pAH06-C2-C8 had colonies.

For this reason the following constructs were decided to be used in all future work:

- pAH06-C2-C7
- pAH07-C1-C4
- pAH09-C2-C5 were

It was decided not to work on pAH10 anymore because of time constraints and because pAH10-C2 wasn't sequence-verified.

08-15/16 Genome integration cassette generation for SS328-leu

Project: Transporter
Authors: Pihla Savola
Dates: 2016-08-15 to 2016-08-16

MONDAY, 8/15

PCR was conducted to generate transporter inserts for genome integration transformation, and set up as follows:

Table6

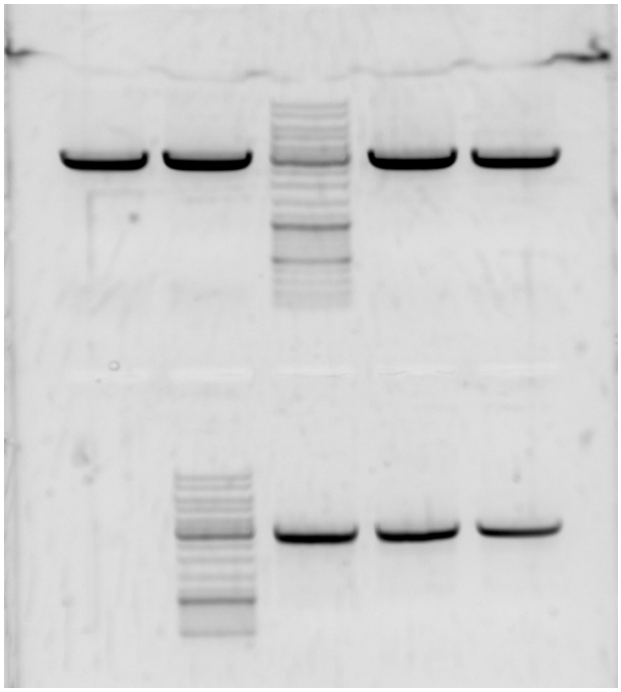
	A	B	C	D	E	F	G
1		pAH06-C1	pAH06-C2	pAH7-C1	pAH8-C2	pAH8-C3	pAH8-C5
2	Phusion Hotstart II Hifi Mastermix (2X)	50 uL	50 uL	50 uL	50 uL	50 uL	50 uL
3	FW primer (PS3, 10 uM solution)	1 uL	1 uL	1 uL	1 uL	1 uL	1 uL
4	RV primer (PS4, 10 uM solution)	1 uL	1 uL	1 uL	1 uL	1 uL	1 uL
5	Template plasmid	0,5 uL (115,2 ng/uL solution)	0.9 uL (60 ng/uL solution)	0.7 uL (73 ng/uL solution)	0.5 uL (115 ng/uL solution)	0.6 uL (95 ng/uL solution)	0.6 uL (96 ng/uL solution)
6	H2O	47.5 ul	47.1 ul	47.3 ul	47.5 ul	47.7 ul	47.4 ul
7	total	100 uL	100 uL	100 uL	100 uL	100 uL	100 uL

Table7

	A	B	C
1	98 C	2 min	
2	98 C	10 s	
3	65 C	30 s	
4	72 C	1 min	cycle to 2, x25
5	72 C	10 min	
6	4 C	hold	

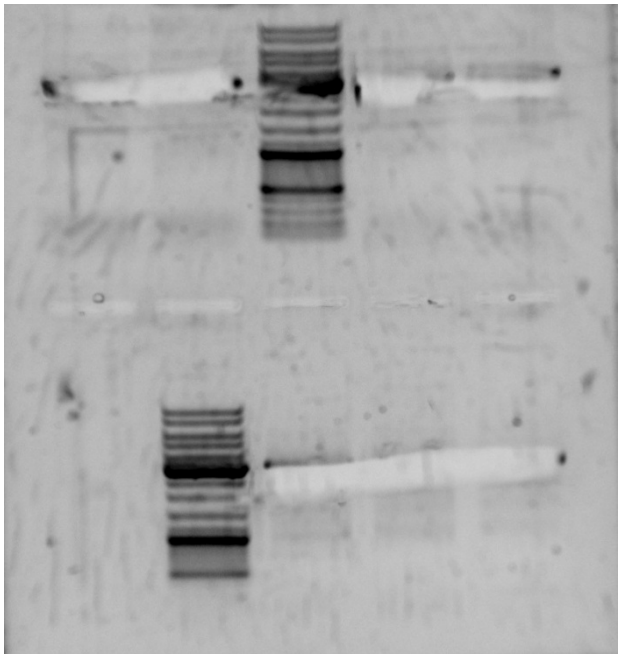
Samples were combined with purple loading dye and run on a 0.7 % agarose gel. Gels were visualized and the identified correct PCR products were excised under UV light. Gel images before and after cut are attached below.

clipboard_2016-08-16_14:01:14.png



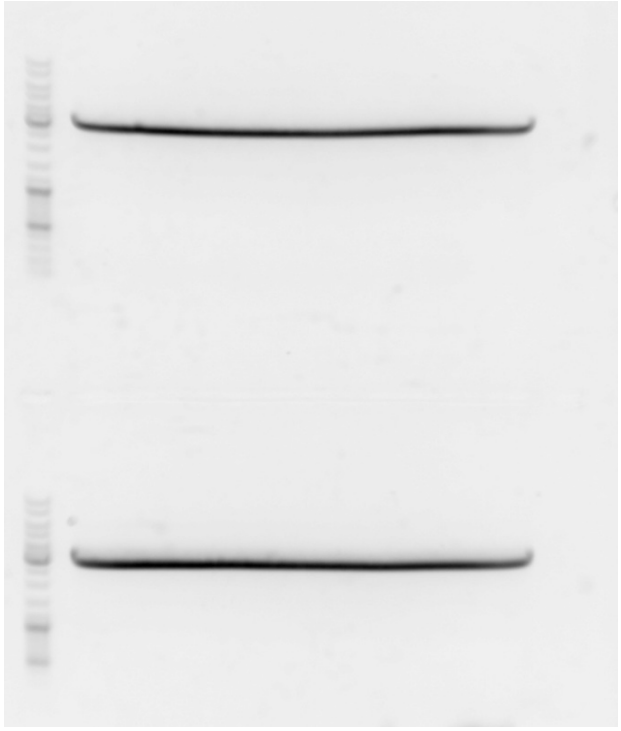
From top left: pAH06-C1 (2 wells), marker, pAH06-C2 (2 wells); bottom left, marker, pAH08-C1 (3 wells)

clipboard_2016-08-16_14:03:11.png



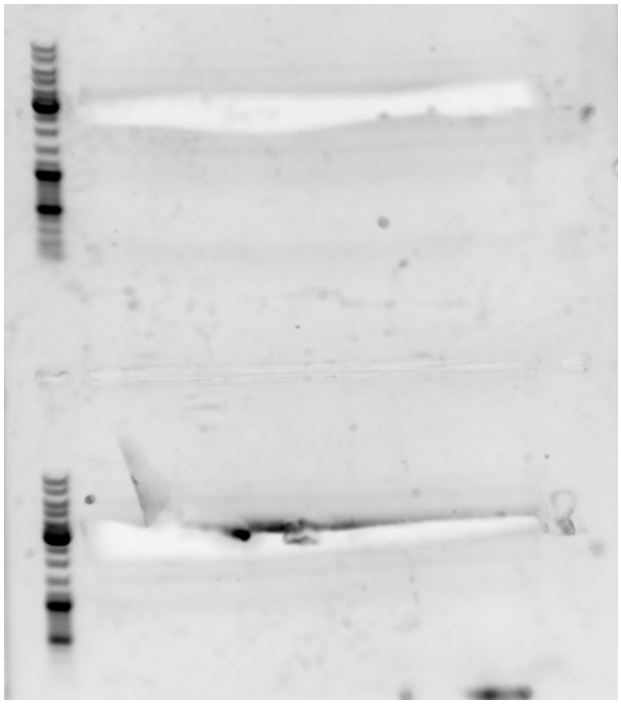
From top left: pAH06-C1 (2 wells), marker, pAH06-C2 (2 wells); bottom left, marker, pAH08-C1 (3 wells)

clipboard_2016-08-16_14:03:49.png



Top left: marker, pAH08-C3, negative control; bottom left, marker, pAH08-C5

clipboard_2016-08-16_14:04:34.png



Top left: marker, pAH08-C3, negative control; bottom left, marker, pAH08-C5

The remaining PCR products were stored at +4 C to be run on the gel on the next day. Additionally, one more PCR reaction was set up, as follows;

Table1

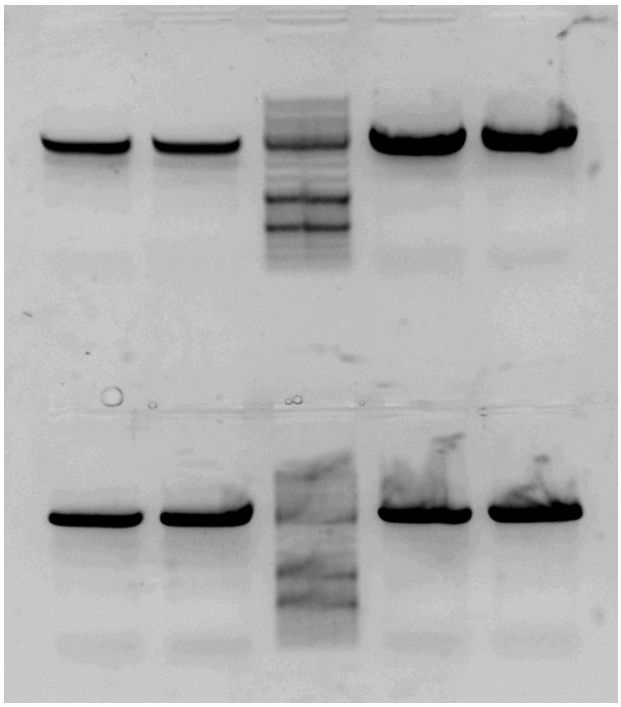
	A	B
1		pAH06-C1
2	Phusion Hotstart II Hifi Mastermix (2X)	50 uL
3	FW primer (PS3, 10 uM solution)	1 uL
4	RV primer (PS4, 10 uM solution)	1 uL
5	Template plasmid	0,8 uL (63,6 ng/uL solution)
6	H2O	47.2 ul
7	total	100 uL

Cyclin conditions were as before.

TUESDAY, 8/16

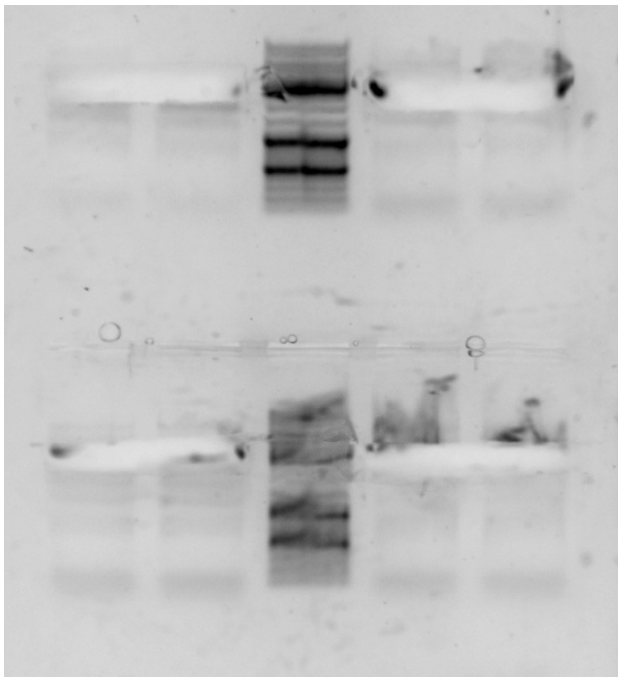
Remaining samples were run on an agarose gel as before. An image before and after excising the desired band is attached below.

clipboard_2016-08-16_14:05:09.png



From top left: pAH07-C1 (2 wells), marker, pAH09-C2 (2 wells); bottom left: pAH10-C1 (2 wells), marker, pAH10-C2 (2 wells)

clipboard_2016-08-16_14:06:40.png



From top left: pAH07-C1 (2 wells), marker, pAH09-C2 (2 wells); bottom left: pAH10-C1 (2 wells), marker, pAH10-C2 (2 wells)

Gel purification was carried out for today and yesterday's samples using the GeneJet Gel Extraction Kit (ThermoFisher) and attached protocol. Elution was done to a volume of 30 uL and final concentrations were measured with Nanodrop.

08-18/19 VL3, W303alpha, SS328-leu Catalase Assay

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-08-18 to 2016-08-19

THURSDAY, 8/18

5 mL liquid cultures were prepared into YPD from W303a, VL3 and SS328-leu.

New solutions of microcystin were made using glass pipettes from methanol extract containing 50 ug/mL. Solutions were prepared to be 2X of the desired sample concentration, as final samples are made with 1 : 1 cell suspension and toxin solution.

Table2

	A	B
1	Final intended MC concentration in samples (ug/L)	Prepared MC solution (ug/L)
2	0	0
3	1	2
4	10	20
5	100	200
6	1000	2000

Solutions were prepared by mixing 0.4 mL of the stock MC extract (50 ug/mL) with 9.6 mL of water to prepare 2000 ug/L solution. Serial dilutions of 200, 20, and 2 ug/L were made by combining 1 mL of the previous solution with 9 mL of water.

FRIDAY, 8/19

Liquid cultures were centrifuged 5 min/3900rpm, supernatant was discarded and cells were diluted into 5ml of 2xPBS.

OD600s from were measured and dilutions were made to reach an OD600=7.

Table3

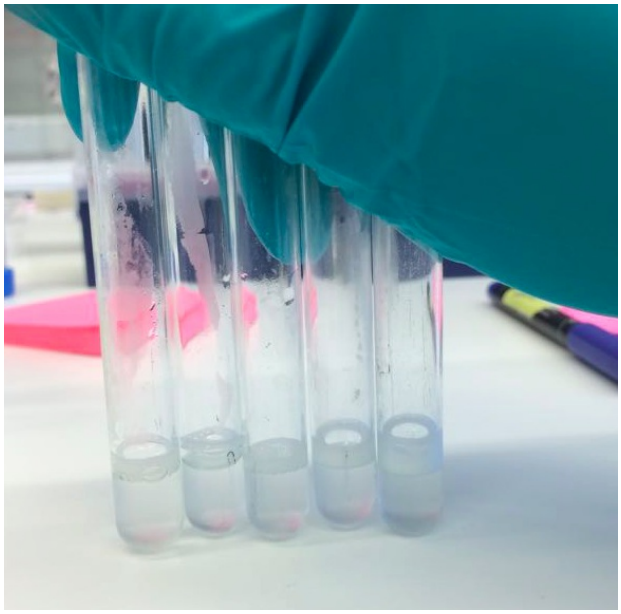
	A	B	C	D	E
1		OD600 (1:10 dilution)	OD600 (1:20 dilution)	OD600 real	dilution to reach OD = 7
2	W303a	1,2	0,69	14	1:1
3	VL3	2	0,92	18	1:1,6
4	SS328-leu	1,1	0,69	14	1:1

Transporters were tested according to the attached protocol with H_2O_2, but the volumes were reduced to half of the described volumes. 0,25 ml of cell solution and 0,25 ml of 2X MC solution (concentrations presented in table 2) was pipetted into glass tubes and incubated in RT for 1h. 4 different toxin concentrations were tested as well as the sample without any toxin. Only the 2000 ug/L toxin solution was mixed prior to pipetting

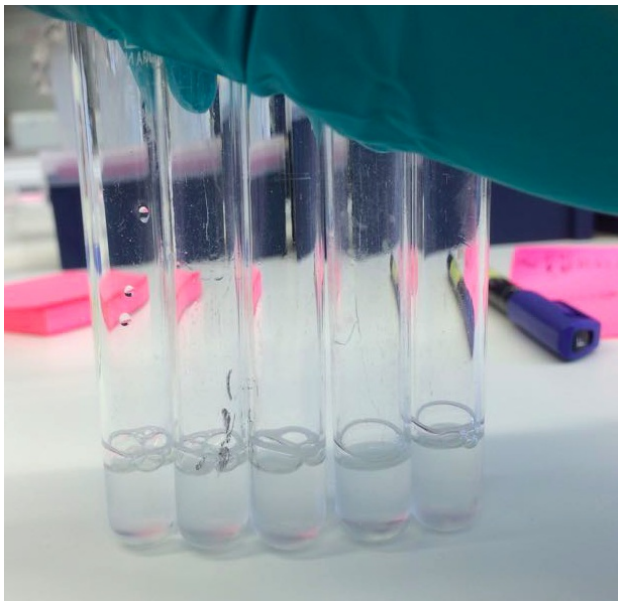
After the incubation, 0,25 ml of H2O2 and 0,1 ml of TritonX-100 were pipetted into each tube. After 5 minutes picture was taken from tubes.

Microcyctin concentration in tubes are 0, 1, 10, 100, 1000 ug/l.

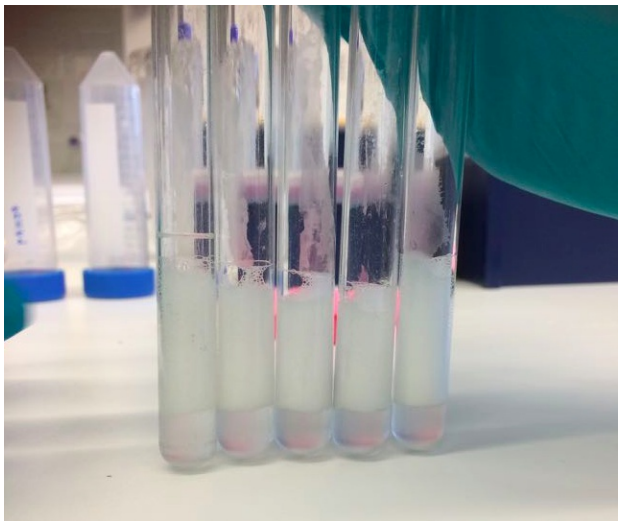
W303a.png



SS328-leu.png



VL3.png



Against our expectations less foam was formed in SS328-leu than in W303a. This should have been other way around as W303a has a mutation that makes it more sensitive to peroxide, as a transcription factor involved in activating stress response genes isn't activated as efficiently. The foam layer in VL3 was thick as expected.

In w303a and ss328-leu there is no variation in foam quantities with different toxin concentrations. In VL3 layers form a U-letter, but this can be within the error marginal.

It is possible that the MC solutions didn't contain the supposed amounts of MC, in spite of proper handling; the stock of MC methanol extract had changed color from a dark brownish-green to yellow since the time it was originally prepared and verified to contain MC.

For this reason, the test should be repeated with a new extract, but if that doesn't produce good results either, a different assay for catalase should.

NO NEED TO MENTION THIS IN THE WIKI SINCE EXTRACT PROBABLY DIDN'T HAVE NO MC IN IT - BUT ACTUALLY THIS GIVES A GOOD INDICATION OF HOW INACCURATE THE MEASUREMENT IS (LOOK AT VL3, technically those all should be identical, except for methanol entry)

08-24/30 pAH09 expression test

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-08-24 to 2016-09-01

WEDNESDAY, 8/24

Overnight liquid culture was prepared of pAH09-C2-C5 colony, which had been confirmed to contain the new transporter. 5 mL of YPD medium in a 50 mL falcon tube was inoculated with the remains of the colony.

The culture was grown at 30 C with shaking at 240 rpm, starting at 8pm.

THURSDAY, 8/25

Hardly any growth was visible on pAH09-C2-C5 (OD 0.1), so incubation with shaking was continued. Additionally, 1 mL was also inoculated in 4 mL of fresh YPD medium for a second culture.

At 4pm, as growth still looked limited, both cultures were pelleted and resuspended in 5 mL fresh YPD medium for incubation overnight, again at 30 C and 240 rpm shaking.

FRIDAY, 8/26

Both cultures appeared cloudy in the morning. After plating 20 uL of b pAH09-C2-C5 on selective Nat+G418 plates, OD was measured for both cultures

Table2

	A	B	C
1		OD600 (1:5)	OD600 (1:10)
2	pAH09	1,299	0,662
3	pAH09_refreshed	1,25	0,727

Cultures were centrifuged 5min at 3900rpm. A sample of the supernatant (S1) was collected before discarding it. Pellets were stored on ice.

Lysis was carried out according to the attached protocol. Pellet was suspended in 300 uL autolaved water, transferred into eppendorf tube and centrifuged (5000g, 5min, +4°C). This washing step was repeated and liquid discarded.

After wash, cell pellets were resuspended in 300 uL 1x SDS-PAGE sample buffer containing 1 mM PMSF and 1x protease inhibitors:

Table1

	A	B	C
1		Amount used	Final concentration
2	4x SDS-PAGE sample buffer	75 uL	1x
3	25x protease inhibitor stock in H2O	12 uL	1x
4	100 mM PMSF (in EtOH)	3 uL	1 mM
5	H20	210 uL	-
6		300 uL	

A 60 uL sample of unlysed cells (UL) was collected at this point before carrying on with the lysis.

Once lysed cells were spun down, a 60 uL sample of the supernatant (S2) was collected. Supernatant and glass beads were then removed and the pellet was resuspended in 240 uL of 1x SDS-PAGE sample buffer; 60 uL was collected for the pellet sample (P2).

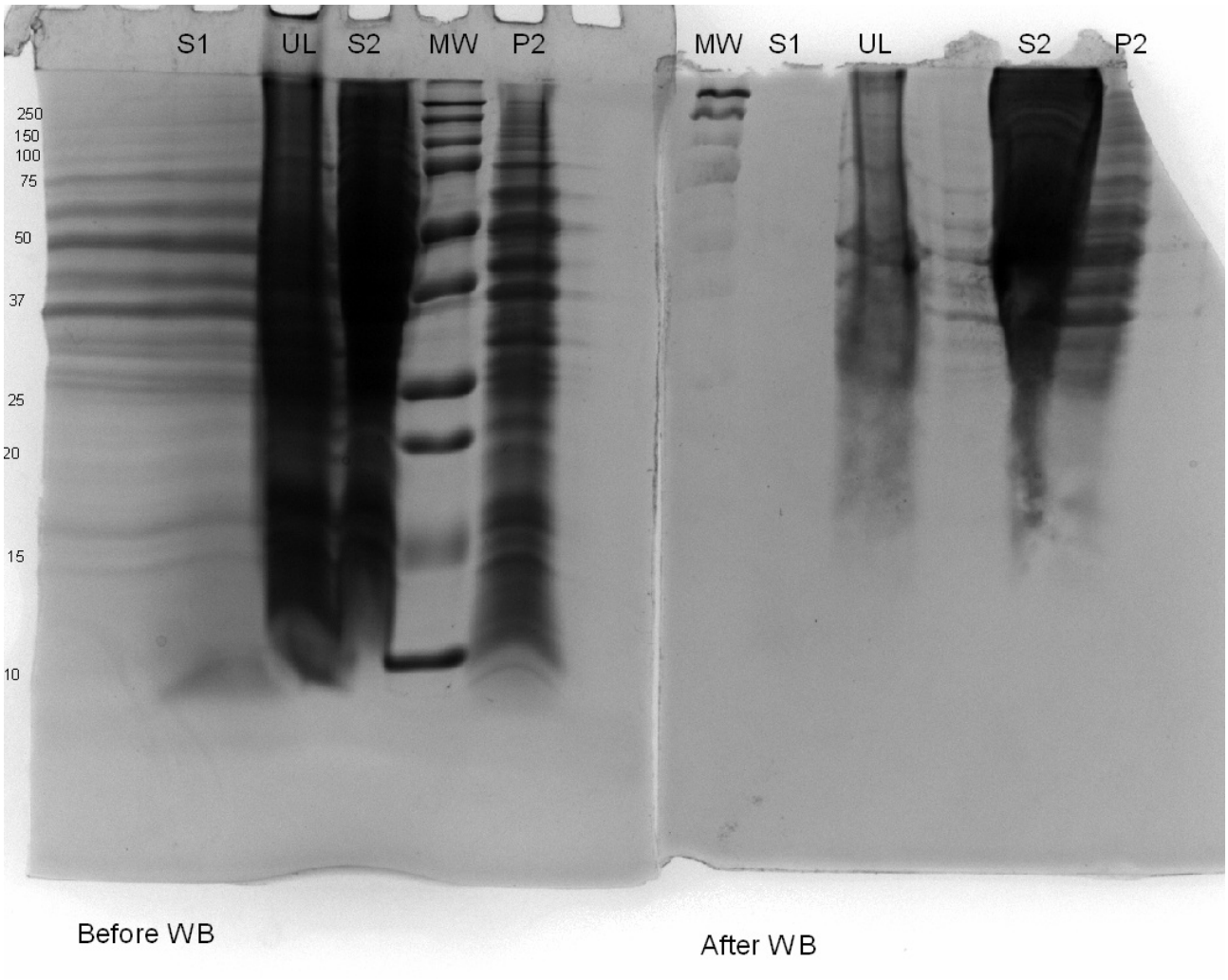
All samples (S1, UL, S2, P2) were heated at 70 C for 5 min, and then loaded on the SDS-PAGE gel. (3%+12.5%).
Around 25 uL of each sample was loaded twice on the gel, in the following order:
-MW, S1, UL (empty), S2, P2, (empty wells), S1, UL, S2, MW, P2

Gels were run at 90 V, 30 mA for 30 min, then voltage was increased to 110 V and gels were run until the loading dye had run through 80 % of the gel.

After the gel was run, it was cut in half so that each half contained all of the samples. With one half, western blot was conducted according to the attached protocol. The other half was stained with coomassie brilliant blue dye (1x used) for 1 hour, then transferred to destaining solution. After blotting, the blotted half of the gel was also stained in the same way.

After sufficient destaining was acheived, (around 2 h), gels were imaged:

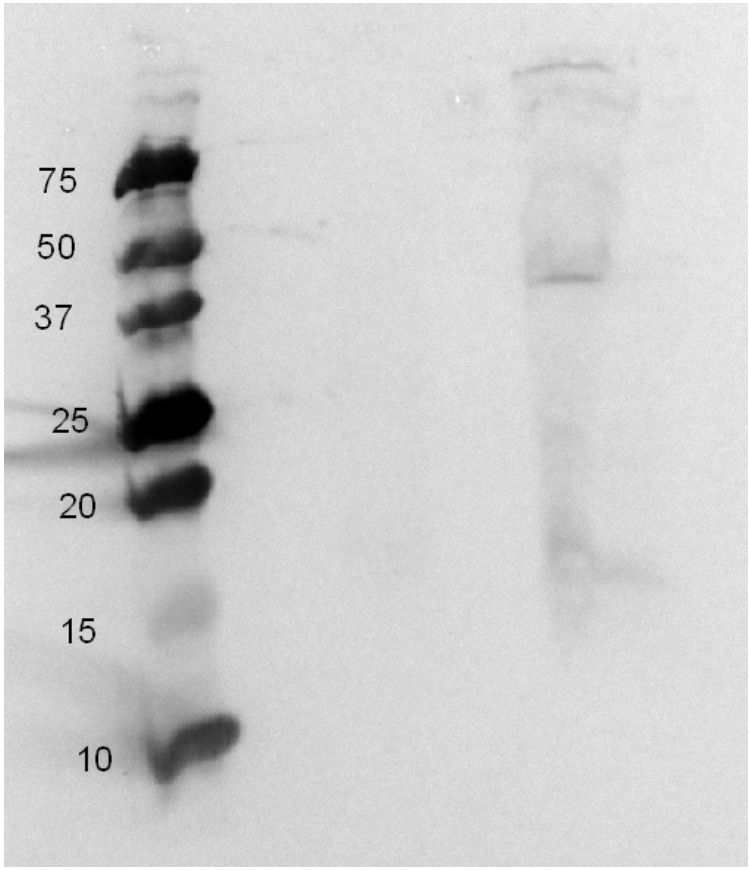
clipboard_2016-08-30_17:32:14.png



Although samples were suspended in volumes recommended for SDS-PAGE sample preparation (Yeast Cell Lysis protocol), the UL sample and especially the S2 sample appear to have too much protein to make out bands. However, in the P2 (pellet) fraction, a band is observed between 37 and 50, which might be the right size (44 kD).

As the blotted membrane was also imaged, a similar band is seen:

clipboard_2016-08-30_17:31:28.png



However, this band would appear to be in S2, not P2, although it is the right size (between 37 and 50 - around 44). It might be possible that the proportions are slightly distorted on the blotted membrane, but a redo of the SDS-PAGE, staining and blotting with less concentrated S2 could confirm the result.

Additionally, it was later noted that TBS-Tween buffer was in fact 2x (containing double the concentration of all the components). Something strange had apparently also happened in preparing TBS-blocking buffer, it is possible that this buffer was a more dilute version of what it should have been (2/5) of the intended.

TUESDAY, 8/30

The SDS-PAGE was redone for the pAH09-C2-C5 cell pellet stored in -20 C. The pellet was resuspended in 300 uL 1x DS-PAGE sample buffer containing protein inhibitor and 1 mM PMSF. Glass beads were added, then tube was vortexed for 10 minutes at +4 C. Sample tubes were centrifuged, 100 uL of supernatant sample was removed, and then the remaining supernatant and glass beads were scooped out. The remaining pellet was resuspended in 200 uL of the sample buffer, and a 100 uL sample was taken out.

The two samples (S and P) were heated for 5 min at 70 C, then briefly vortexed and spun down. 3 different volumes of each sample was loaded twice on the gel, in the following order:

- S (20 uL), P (20 uL), S (15 uL), P (15 uL), S (5 uL), P (5 uL), MW, MW, S (20 uL), P (20 uL), S (15 uL), P (10-15 uL), MW, S (5 uL), P (5 uL)

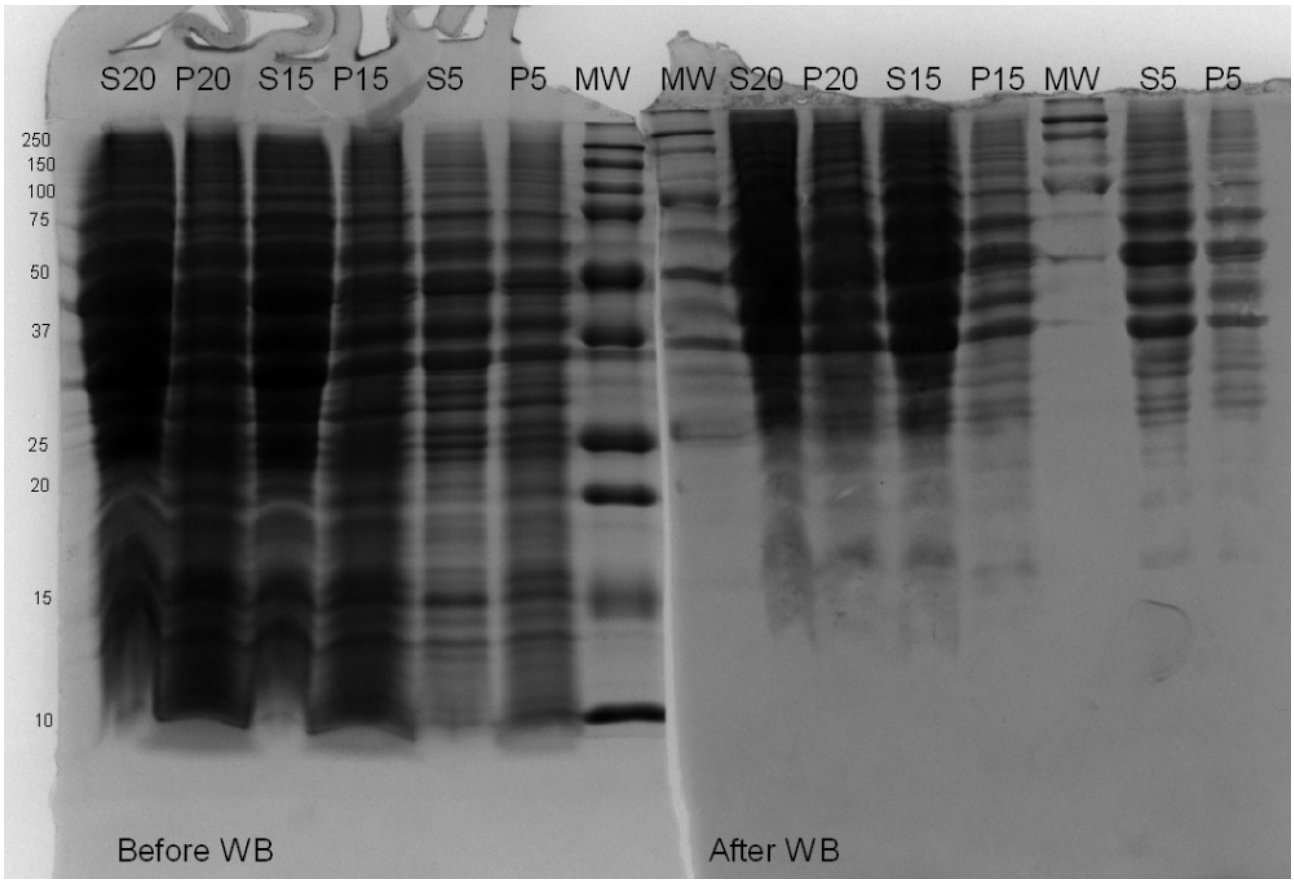
The gel was run at 80 V, 30 mA for 10 minutes, then the voltage was increased to 110 V and the gel was run for 50 minutes. After this, voltage was increased to 200 V until the dye was run around 80 % of the gel.

After the run, the gel was cut in half between the two MW lanes; one half was transferred to a nitrocellulose membrane as before, the other stained with coomassie blue stain (1x used, 1 hour with shaking).

After blotting, visualization of the blot followed according to the Strep-Tactin AP conjugate blot protocol. The half of the gel used for blotting was stained with coomassie blue stain as before. Destaining was for 2-3 hours.

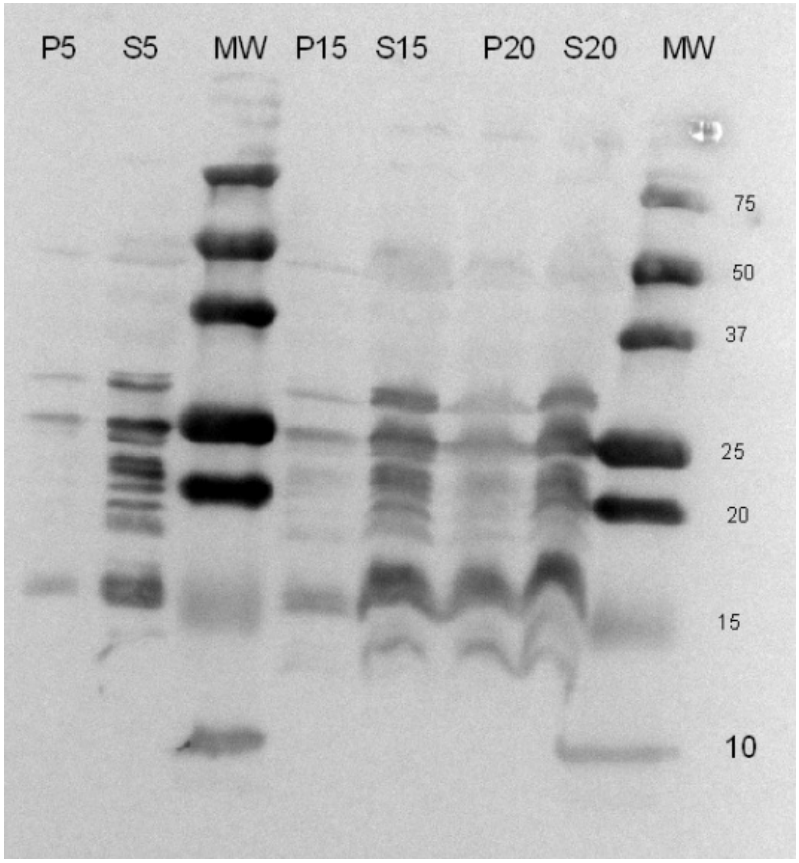
Imaged coomassie stained gels:

clipboard_2016-08-30_17:34:12.png



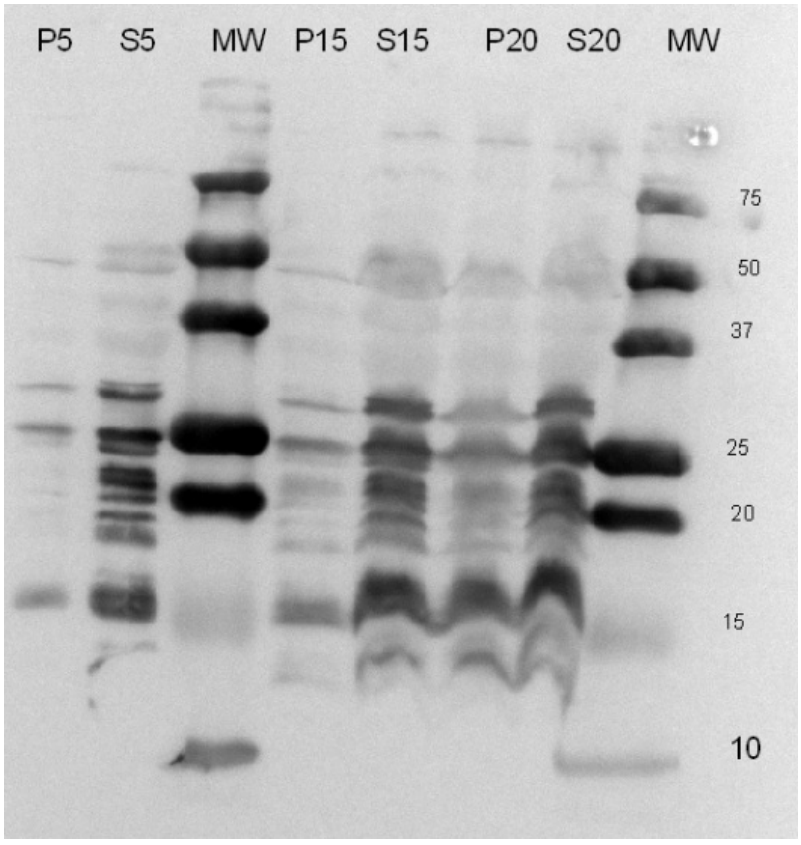
Color development for NBT/BCIP treatment of transfered blot:

clipboard_2016-08-30_17:34:54.png



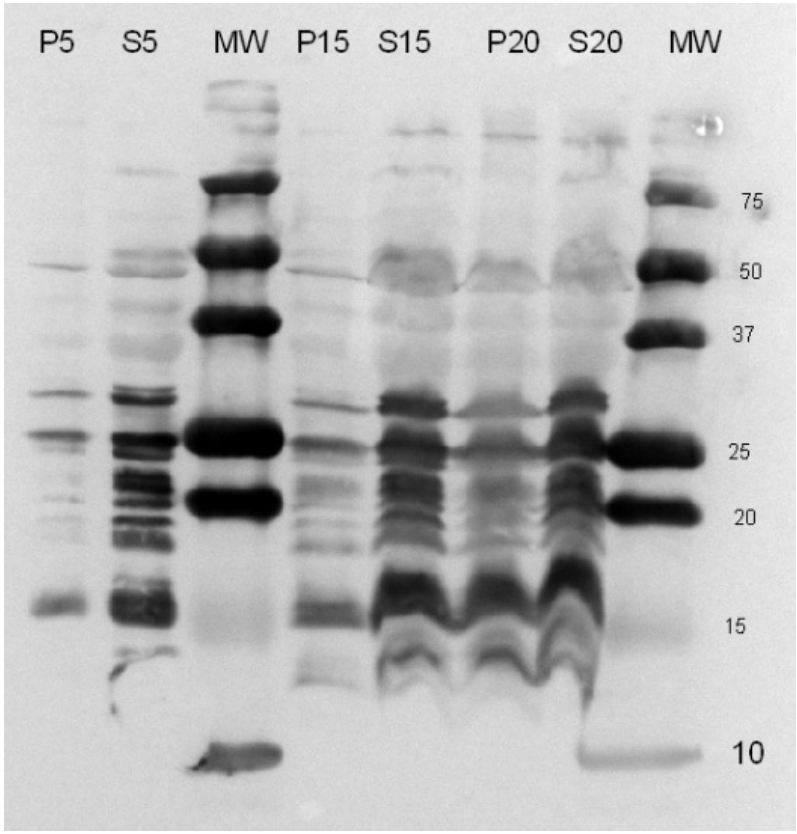
10 min incubation with NBT/BCIP

clipboard_2016-08-30_17:35:14.png



20 min incubation with NBT/BCIP

clipboard_2016-08-30_17:35:36.png



35 min incubation with NBT/BCIP

In both the stain and blot, every sample contains bands between 37 and 50 which could contain the transporter (44 kD), but the blots contain so much unspecific products that nothing conclusive can be said.

It can be noted, however, that most of the proteins in the lower half of the blotted gel have been transferred to the membrane - the top half not as completely, which could explain why there's also more background on the gel's lower half.

In general, it is best to look at the P5 and S5 samples, as they seem to contain the most optimal amount of proteins - this amount should be used if future samples are prepared in a similar way.

Part of the blot's problem could be inefficient and particularly uneven transfer of proteins to the nitrocellulose membrane.

One way to confirm transporter expression would be to do pAH6 as a negative control in parallel with pAH9, as the only difference between the constructs is the absence of strep-tag on the pAH6 transporter. This way, information about background proteins could be obtained, and the strep-tagged transporter band could be obtained with more certainty.

WEDNESDAY, 8/31

5 mL precultures in YPD were inoculated for pAH09-C2-C5, pAH06-C2-C8 and SS328-leu

THURSDAY, 9/1

No growth was visible for pAH06-C2-C8, so only pAH09-C2-C5 and SS328-leu were processed further. ODs were measured for the cultures:

Table3

	A	B	C
1	dilution	SS328-leu	pAH09-C2-C5
2	1:10	0.693	0.397
3	no dilution	6.93	3.97

Cell lysis was carried out as before. Centrifuged cell pellets were.resuspended in 1x SDS-PAGE loading dye with 1x protease inhibitors and 1 mM PMSF; SS328-leu in 170 uL, pAH09-C2-C5 in 100 uL. Glass beads were added, then tube was vortexed for 10 minutes at +4 C. Sample tubes were centrifuged, 50 uL of supernatant sample was removed, and then the remaining supernatant and glass beads were scooped out. The remaining pellet was resuspended in the same volume of the sample buffer, and a 50 uL sample was taken out.

The two samples (S and P) were heated for 5 min at 70 C, then briefly vortexed and spun down. 5 uL of each sample was loaded four times on the gel.

After running the gel, a pieace of the gel containing all samples once was cut out for coomassie staining; the rest was blotted according to the attached protocol. The blotted membrane was divided into three parts, all of which contained each sample once. Each part of the membrane was washed and processed with washing buffer (TBS-Tween) of a different composition:

Table4

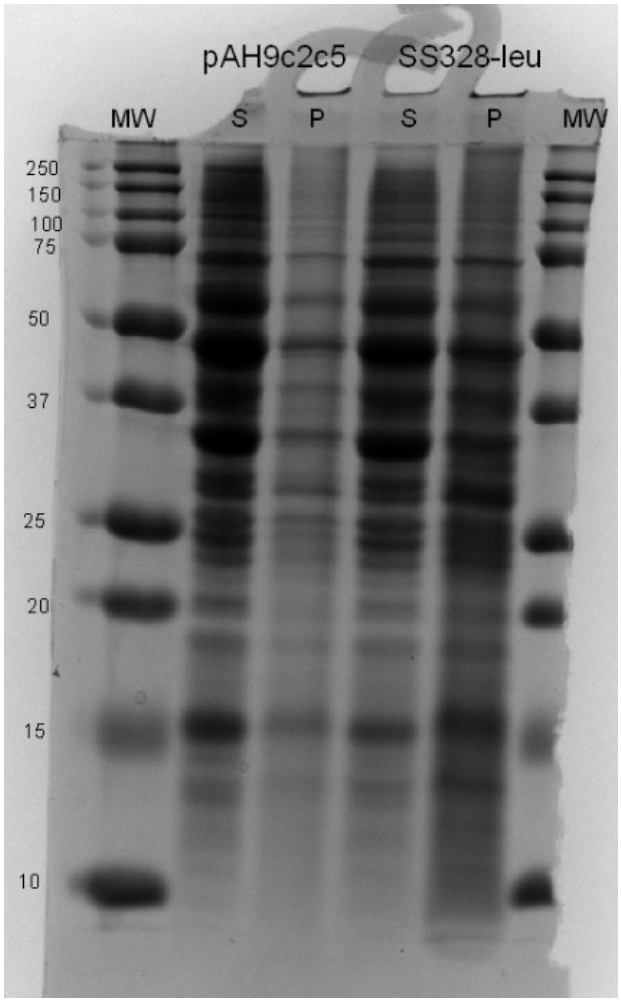
	A	B	C	D	E
1	Membrane part number	Tris (mM)	NaCl (mM)	Tween 20 (w/v %)	pH
2	1	50	140	0.1	7.4
3	2	50	300	0.1	7.4
4	3	50	200	0.2	7.4

Washing times and volumes were extended. 30 min was used as the color development time when visualizing all the blots.

The blotted gel pieces were stained in the same way as the unblotted; 1 hour staining with 1x used coomassie blue stain, then destaining overnight.

The imaged gels and blots are as follows:

clipboard_2016-09-07_13:55:43.png



Unblotted samples

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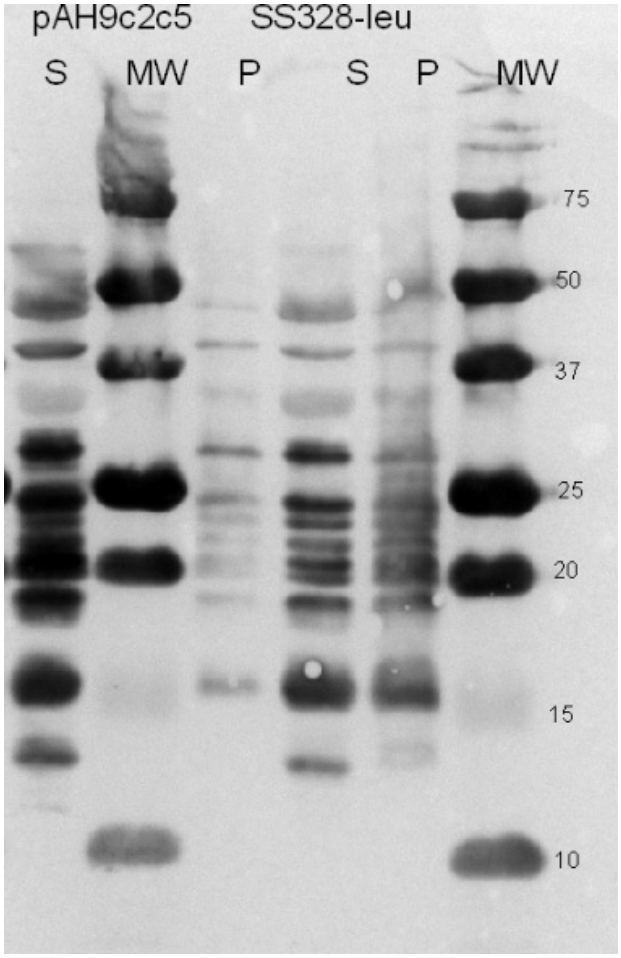
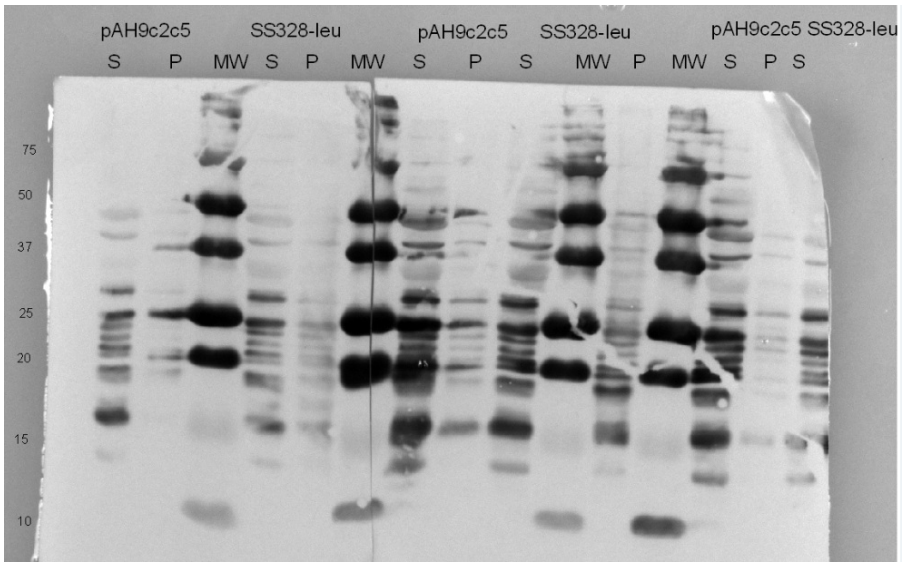


Wash buffer 1 (normal)

clipboard_2016-09-07_13:58:07.png



Left half from the cut: wash buffer 2 (more NaCl) - right side of the cut: wash buffer 3 (more Tween 20)

No differences between control and sample are observed. Because unspecific bands should have been reduced by the different washing procedures, most likely the bands are endogenously biotinylated proteins which stain sensitively. It looks like any correct band, if existing, is faint and obscured by the other bands. It could be that the expression level is too low to be detected in this case; AP conjugated strep-tactin is also much less sensitive than e.g. HRP conjugated.

Higher expression levels and/or blocking of biotinylated proteins would be needed to verify transporter expression. A better functional test to verify transporter functionality would also be useful.

08-29 Transporter assay with H2O2 in SS328-leu

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-08-27 to 2016-08-30

SATURDAY, 8/27

Liquid cultures were prepared from following plates: pAH06-C2-C7, pAH06-C2-C8, pAH07-C1-C4, pAH07-C1-C5, pAH09-C2-C5, pAH10-C2-C1, pAH10-C2-C2, SS328-leu, VL3 in YPD

SUNDAY, 8/28

Liquid cultures were centrifuged 5mins in 3900rpm and then diluted into 5ml of 2xPBS. (for pAH09 two tubes were combined because there was not much cells, even this way OD didn't reach the value of the others.) Cell solutions were diluted to get an OD600=6, with the exception of pAH09-C2-C5, for which such an OD couldn't be be acheived, even though two 5 mL cultures were combined.

Table1

	A	B	C	D	E	F	G
1	construct	OD600	OD600, real	dilutions (cells-PBS)			
2	SS328	0,191 (1:50)	9,55	2,05+0,95			
3	VL3	0,471 (1:50)	23,55	0,76+2,25			
4	pAH06-C2-C8	0,186 (1:50)	9,3	1,94+1,06			
5	pAH07-C1-C4	0,265 (1:50)	12,8	1,41+1,59			
6	pAH09-C2-C5	0,385 (1:10)	3,85	3+0			

Microcystin solutions were prepared from 50 mg/mL methanol extract. The following solutions were prepared:

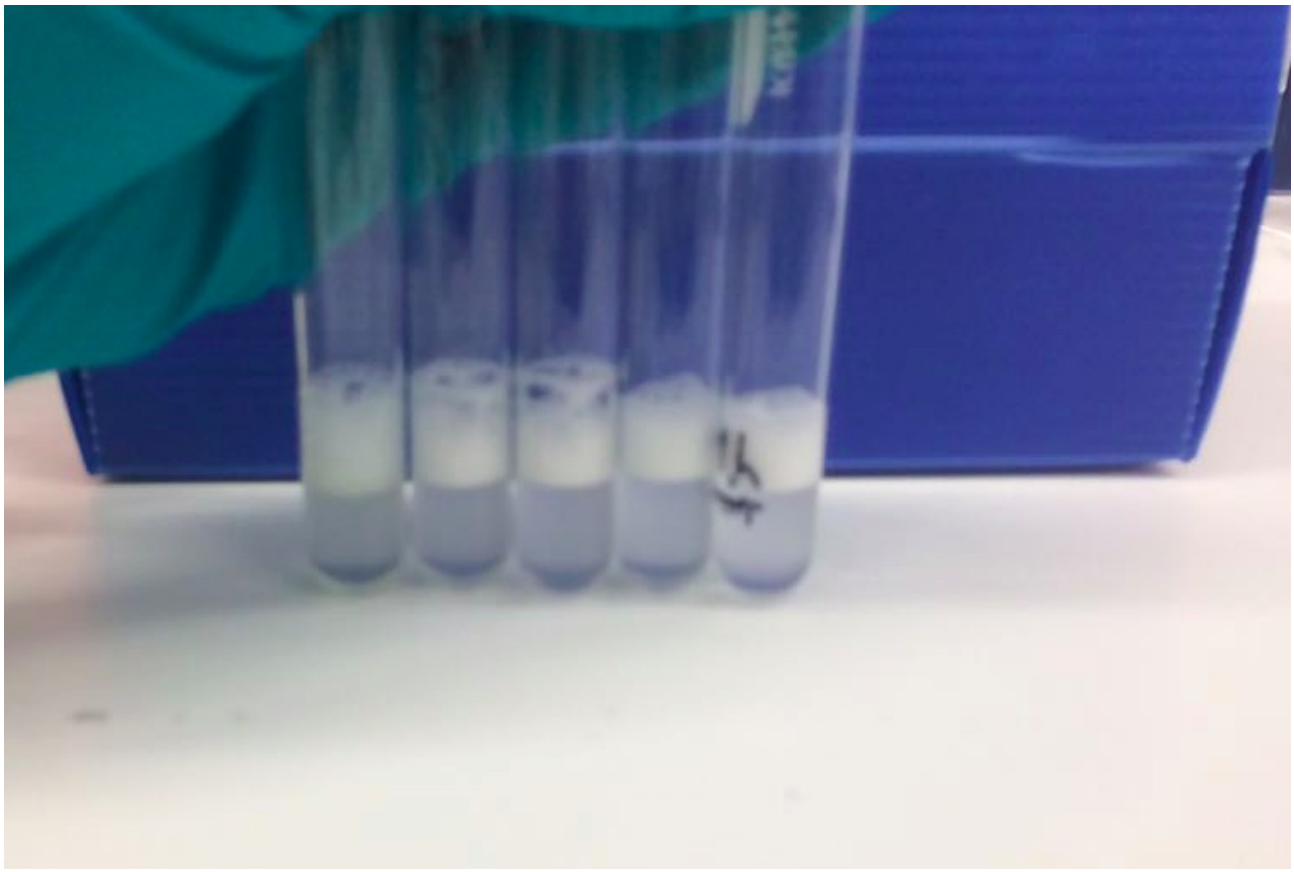
Table2

	A	B
1	Final intended MC concentration in samples (ug/L)	Prepared MC solution (ug/L)
2	0	0
3	1	2
4	10	20
5	100	200
6	1000	2000

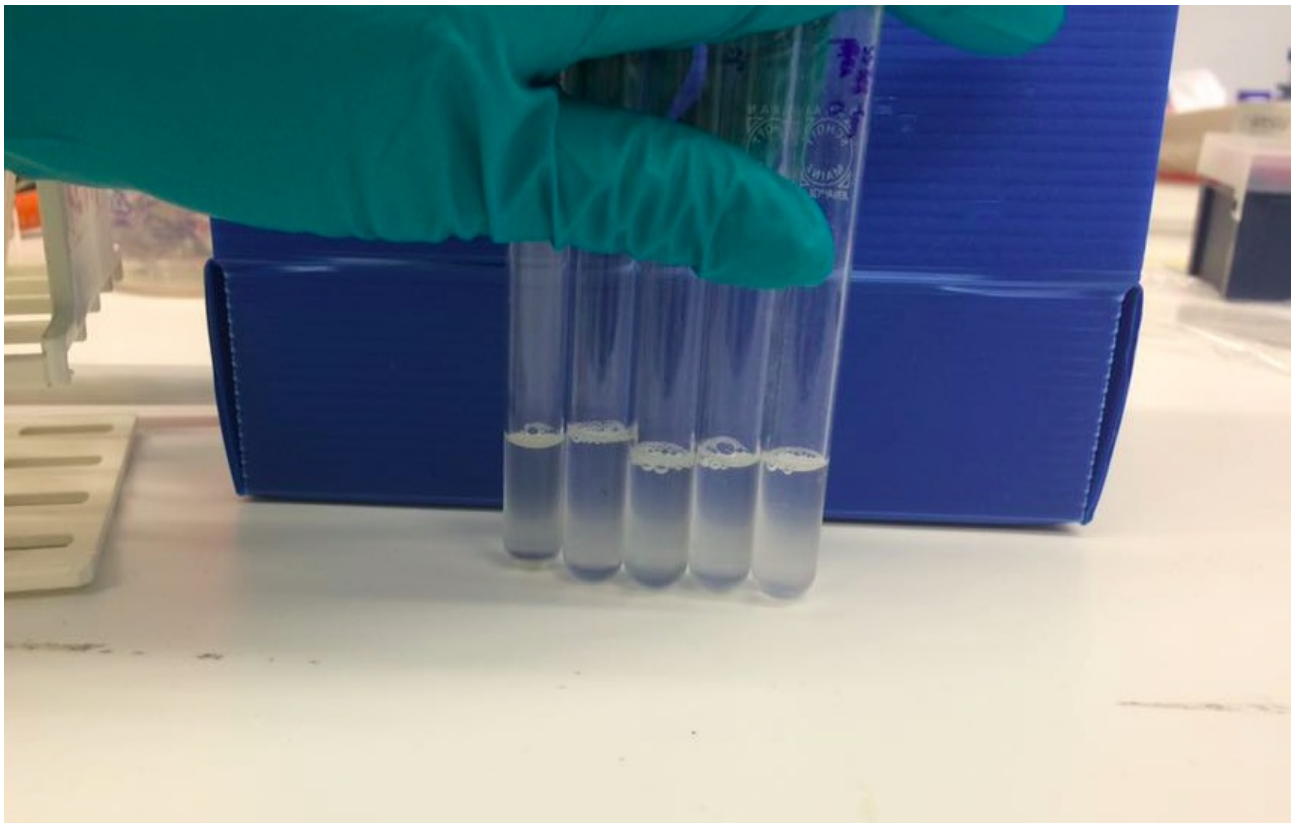
2000 ug/L solution was prepared by mixing 0.2 L of the 50 mg/mL extract with 4.8 mL of water. Serial dilutions were made step by step by mixing 4.5 mL of water with 0.5 mL of more concentrated MC solution.

Catalase test were conducted according to the attached protocol.

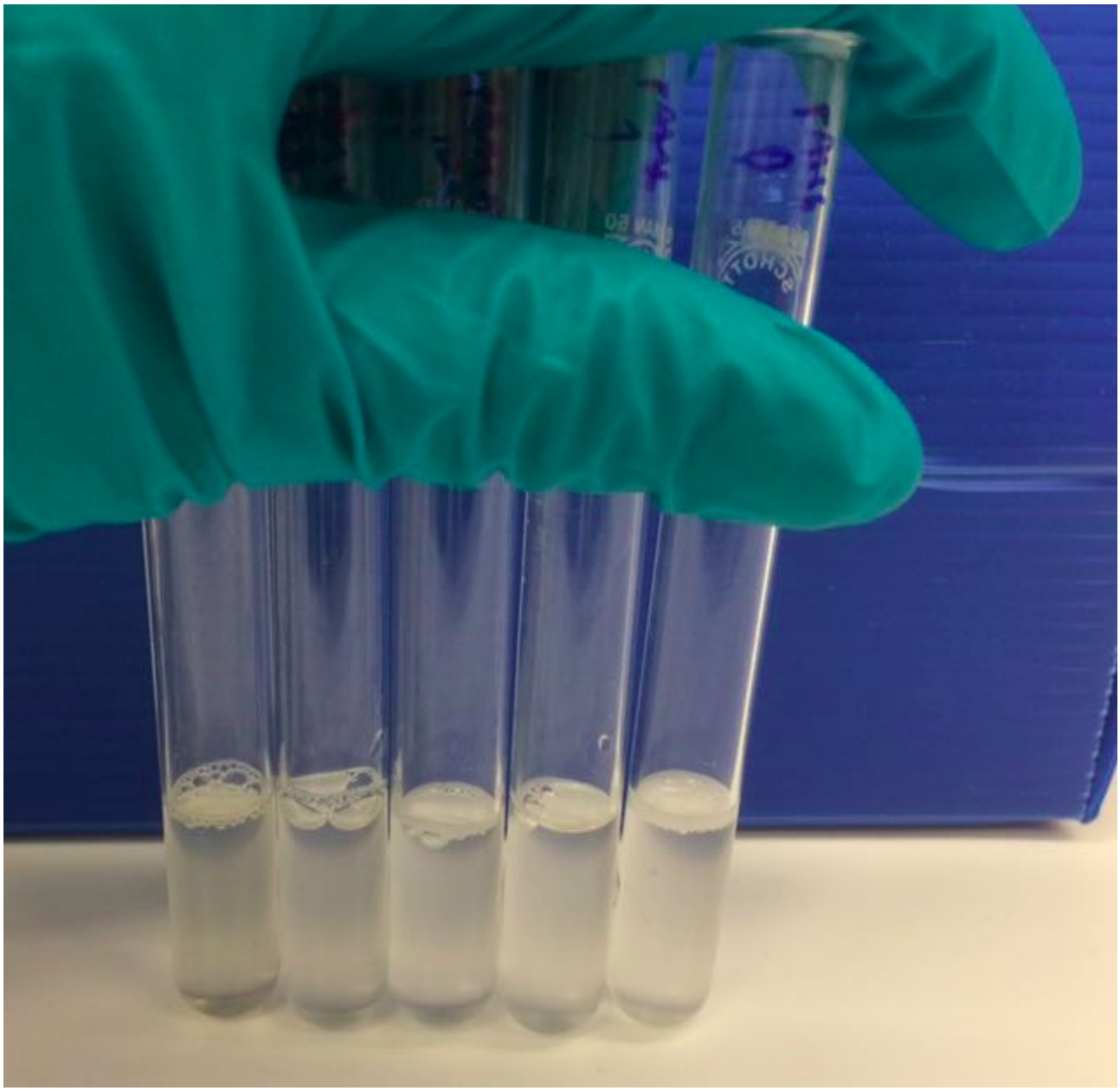
VL3.png



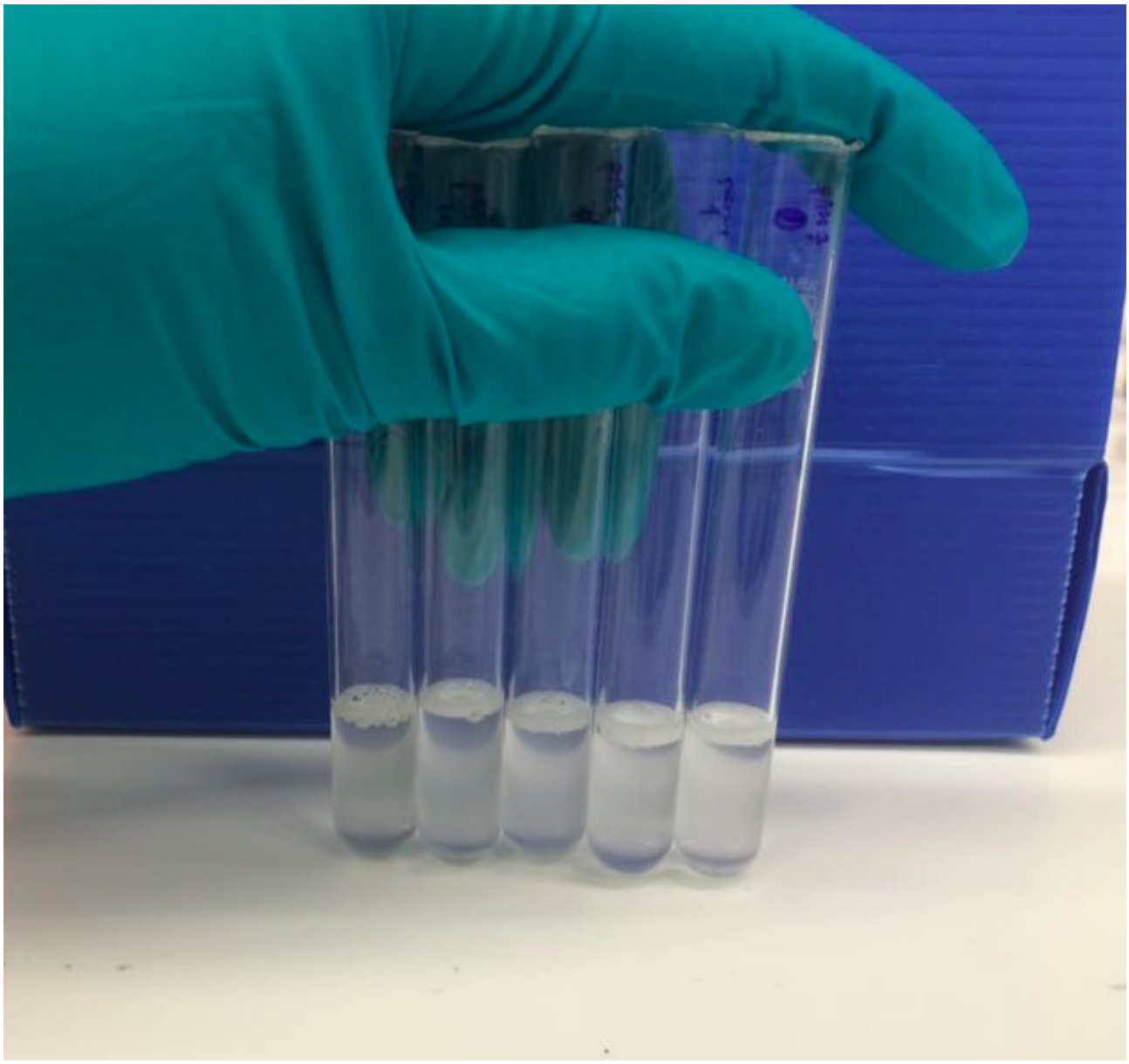
SS238-leu.png



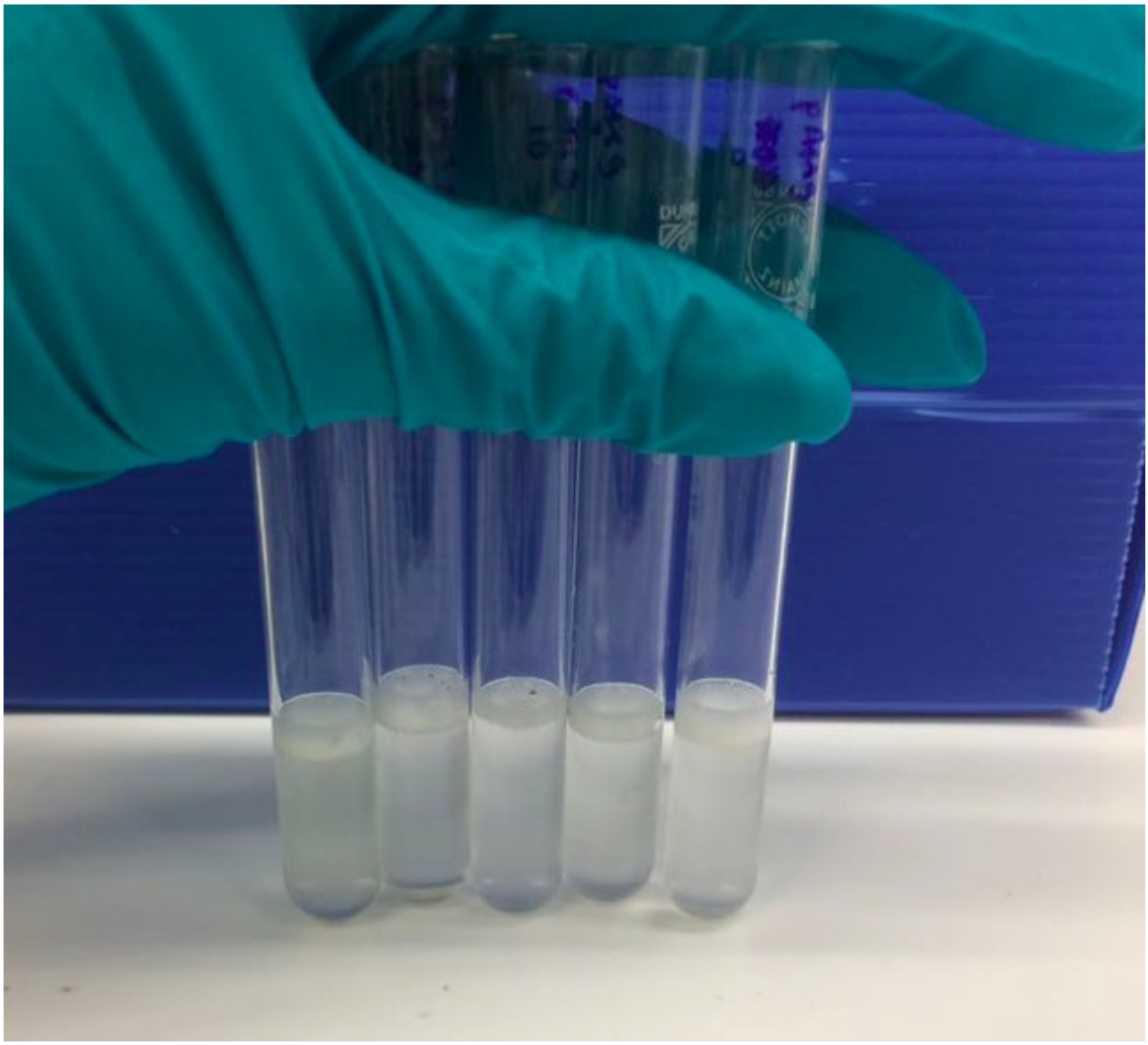
pAH06.png



pAH07.png



pAH09.png



There is no trend to be seen from the following pictures. Microcystin concentrations do not seem to affect the catase activity and foam formation at all. Between inserts and no insert there is though some difference. In SS328 and pAH06 there is basically no bubbles at all while in pAH07 and pAH09 there is a foam layer. There is no good explanation for this as for example pAH06 and pAH09 are the same constructs with the exception that 09 has a strep tag.

NOTE 09/18: In retrospect, the lack of foam in pAH06 could be explained by lack or incorrect incorporation of the new transporter; in later transporter verifications (PCR, restreaking on selective plates), pAH06-C2-C8 didn't appear to have the new transporter after all; the reason could maybe be a mixed colony that originally had only a few copies of no new transporter taking over)

MONDAY, 8/29

New liquid cultures from VL3, SS328-leu and pAH09-C2-C5 were prepared in YPD.

TUESDAY, 8/30

Liquid cultures were centrifuged 5mins in 3900rpm and then diluted into 5ml of 2xPBS. Cell solutions were diluted to get an OD600=3,7 and OD600=1,8.

Table3

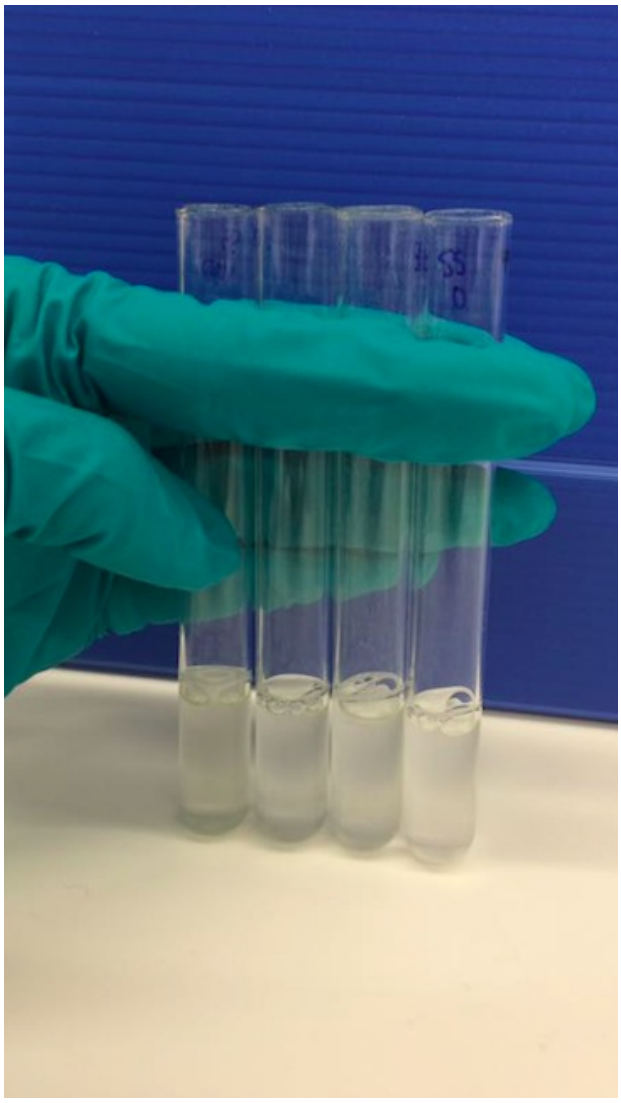
	A	B	C	D	E
1		OD	real OD	dilution (cell+PBS) 5ml tot to OD=3,7	dilution (cell+PBS) 2ml tot to OD=1,8
2	VL3	0,417	20,85	0,887+4,113	1:1
3	SS328	0,206 (1:50)	10,3	1,81+3,19	1:1
4	pAH09	0,373 (1:10)	3,73	5,0	1:1

This time we did catalase test measurements with two different ODs (3,7 and 1,85) and two different time points (1h and 4h). Two different time pointrs were made only for samples with OD values of 3,7.

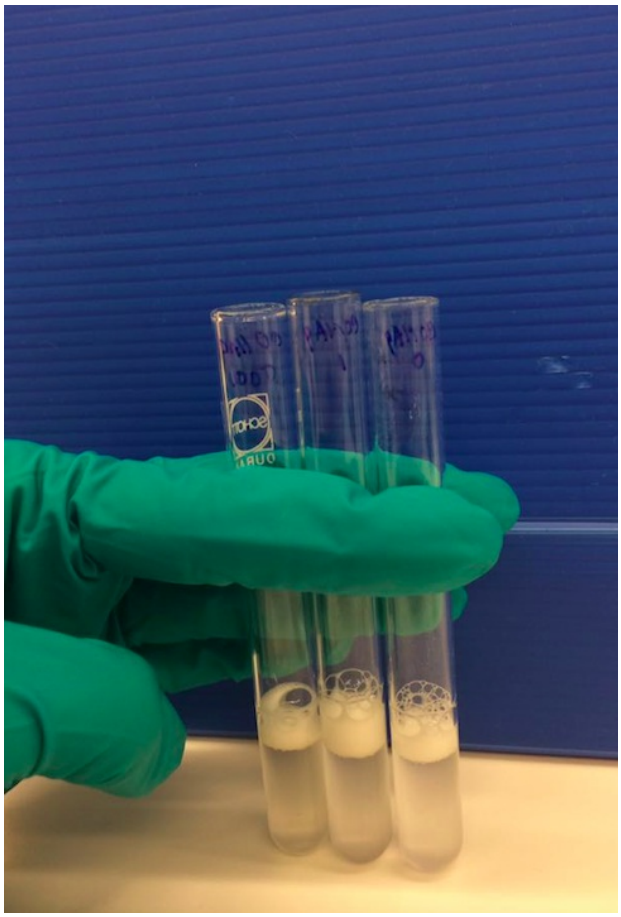
Microcystin solutions were prepared same way as yesterday (100ug/l was not used). Catalase test were conducted according to the attached protocol.

VL3,1h,OD=3,7.png

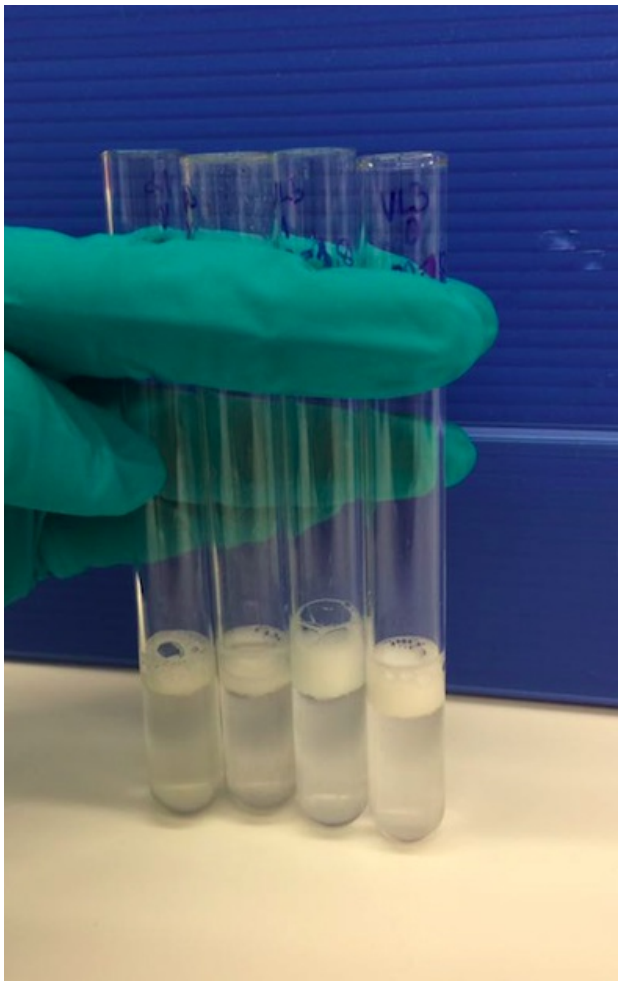
SS328,1h,OD=3,7.png



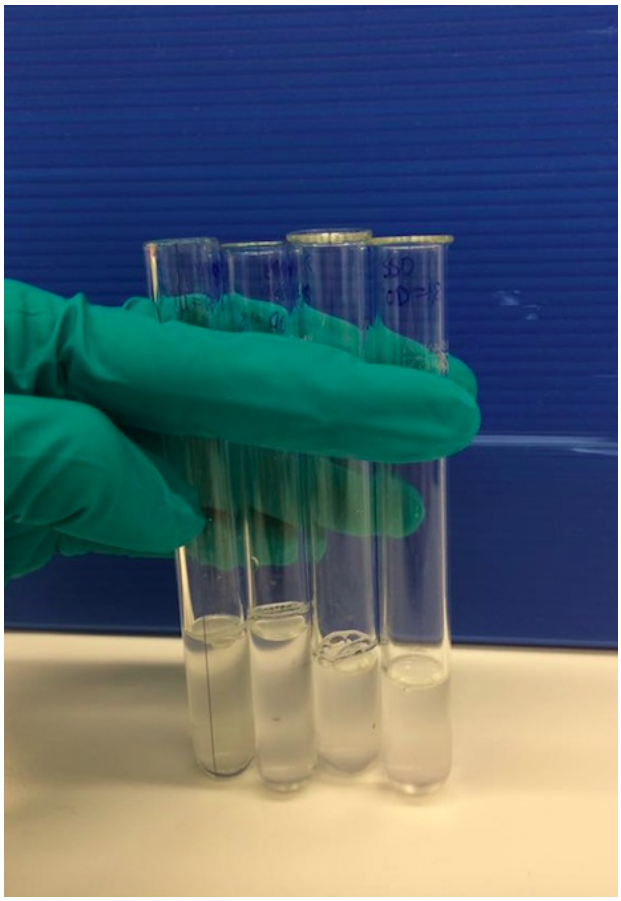
pAH09,1h,OD=3,7.png



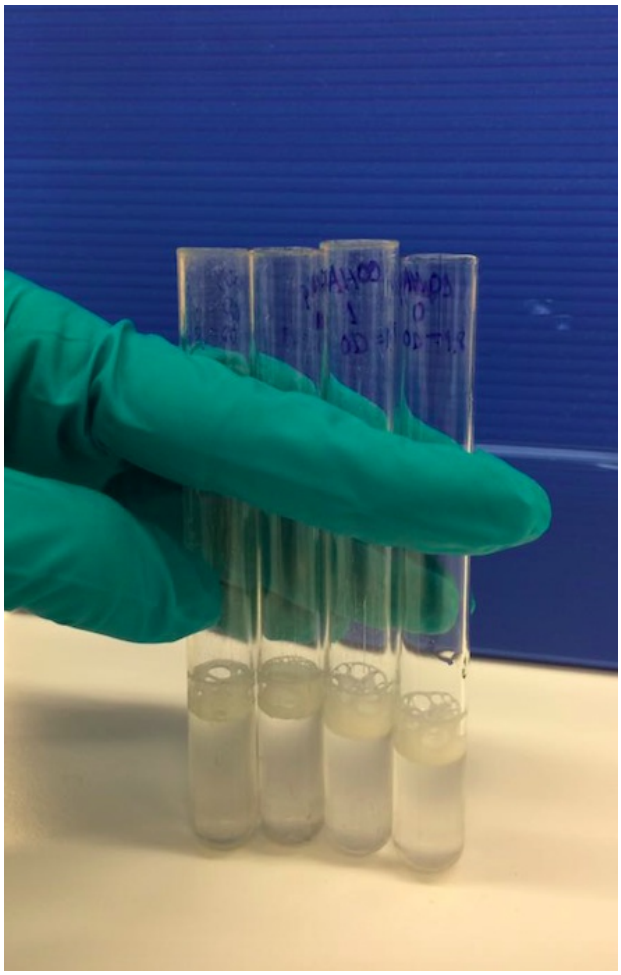
VL3,1h,OD=1,8.png



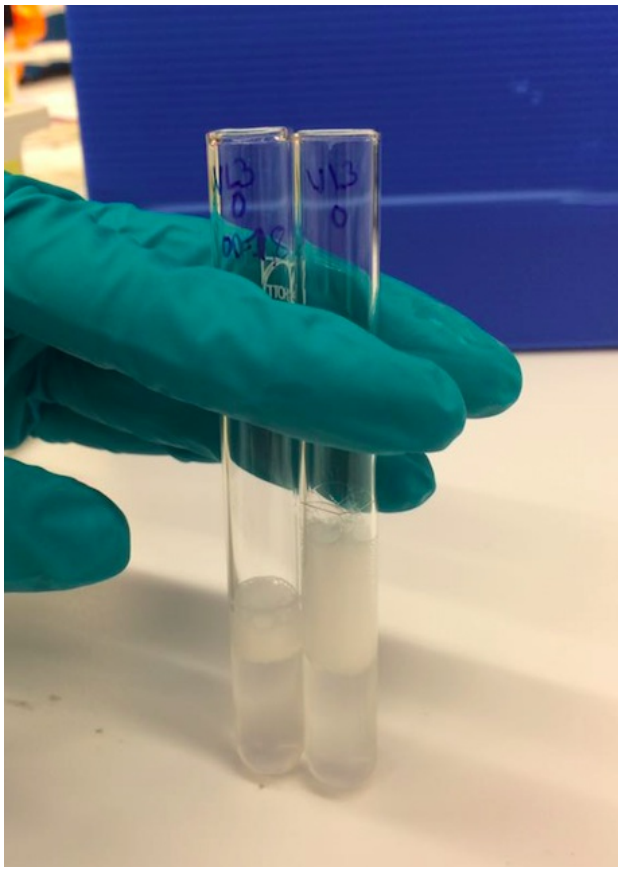
SS328,1h,OD1,8.png



pAH09,1h,OD=1,8.png



VL3, different ODs.png

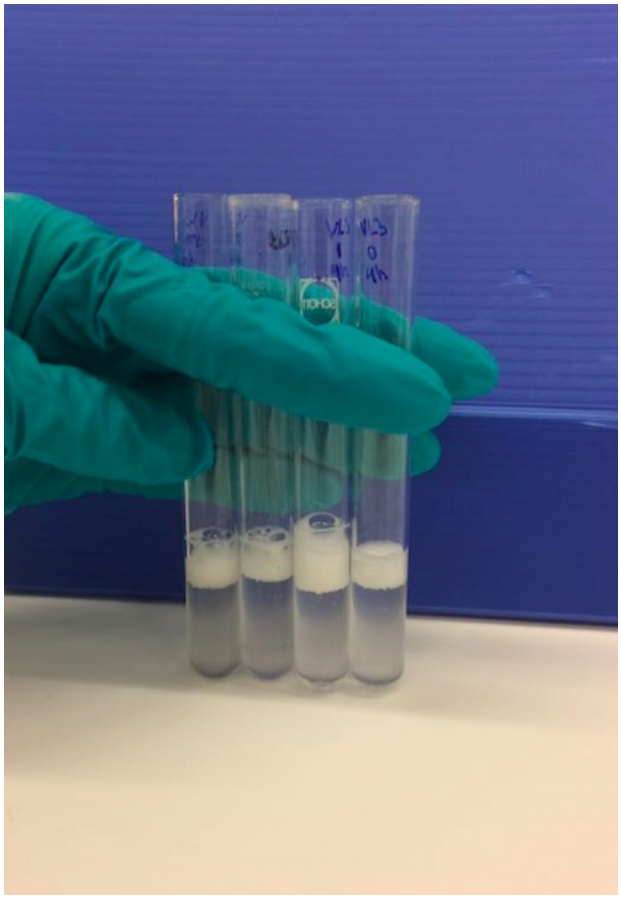


From these results we can see that definitively higher OD is better. This makes sense because the more cells we have, the more catalase they produce.

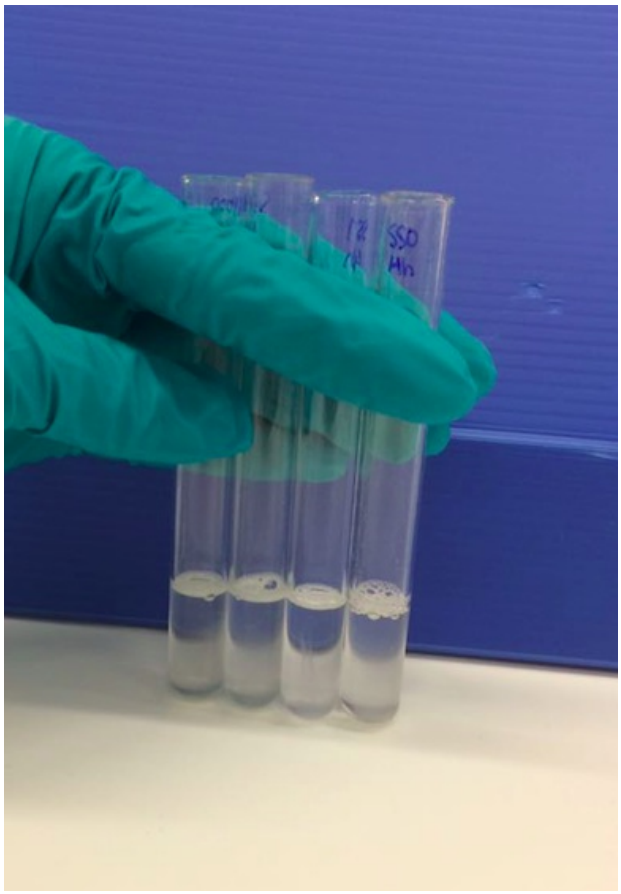
We can also see that pAH09 produces more foam than SS328. This could be due to the transporter as there is no other difference between SS328 and pAH09 (except geneticn resistance). Towards different MC-LR concentrations there is no reaction.

One noticable aspect is that pAH09 grows a lot slower than SS328-leu.

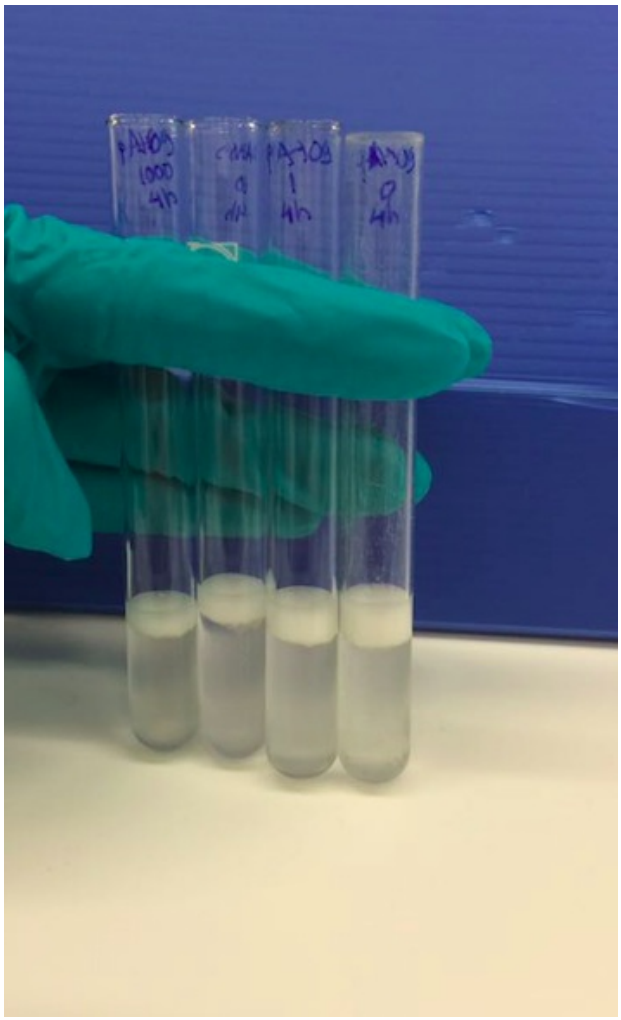
VL3,4h,OD=3,7.png



SS328,4h,OD=3,7.png



pAH09,4h,OD=3,7.png



09-12/13 pAH09+pAH05 Gibson Assembly

Project: Transporter
Authors: Hele Haapaniemi
Dates: 2016-09-11 to 2016-09-15

SUNDAY, 9/11

This entry describes gibson assembly to transfer QDR2I_Cstrep to pRS415-GAL plasmid backbone. This will enable expression of QDR2I_Cstrep in higher quantities, so that hopefully it and its correct expression can be detected with western blot.

Primers PS13-18 and VF2 + VR were resuspended in H2O (100 uM concentration) and 20 uL dilutions with 10 uM concentration were prepared.

PCR was done to get QDR2I_Cstrep insert out of pAH09 plasmid backbone and to add overhangs for gibson assembly into linearized pAH05 plasmid; PCR reaction was set up as follows

Table1

	A	B
1	Phusion HotStart II HiFi Mastermix	50 uL
2	PS17, 10 uM (FW primer)	1 uL
3	PS18, 10 uM (RV primer)	1 uL
4	pAH09-C2 (Template DNA)	0.8 uL (66 ng/uL solution)
5	H20	47.3 uL

Cycling conditions were as follows:

Table2

	A	B	C
1	98	2 min	
2	98	10 s	
3	65	30 s	
4	72	30 s	back to 2, x25
5	72	10 min	

MONDAY, 9/12

pAH05-C10 plasmid was digested with SpeI (BcuI) and XhoI enzymes to prepare it for gibson assembly with the PCR'd insert. Restriction digest was set up:

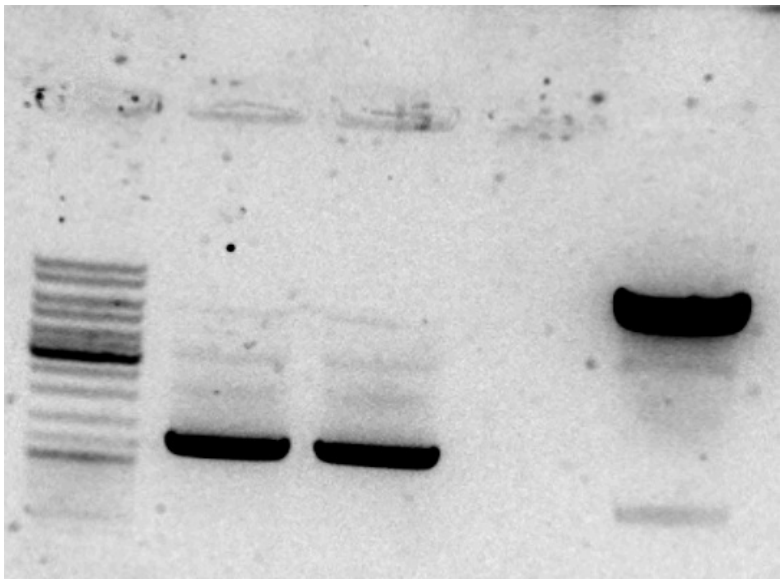
Table3

	A	B
1	pAH05-C10 (template DNA, 268 ng/uL)	8 uL
2	FD buffer	2 uL
3	FD BcuI	1 uL
4	FD XhoI	1 uL
5	H2O	8 uL
6	total	20 uL

Digest was incubated at 37 C for 15 minutes. 4 uL of purple loading dye was added and the digest was loaded on a 0.7 % agarose gel. Yesterday's PCR was also loaded on the gel after adding loading dye (20 uL).

Visualized gel is as follows:

clipboard_2016-09-13_15:09:42.png



(from left; DNA ladder, pAH09-C2 PCR product in 2 wells, empty well, pAH05-C10 RD product)

The correct bands are 1260 bp for the pAH09-C2 PCR product and 6658 bp for the pAH05-C10 restriction product. The identified bands were cut out and the gel was imaged after the cut.

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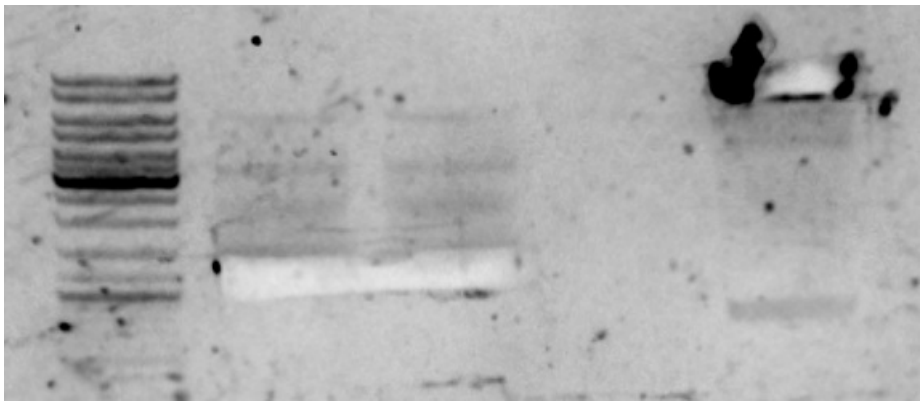


(from left; DNA ladder, pAH09-C2 PCR product in 2 wells, empty well, pAH05-C10 RD product)

The excised bands were purified using GeneJet Gel Extraction kit (ThermoFisher) and the attached protocol. Elution was carried out to a volume of 30 uL, and DNA concentrations were determined using Nanodrop.

A Gibson Assembly reaction was set up as follows, according to the attached protocols:

Table4

	A	B
1	NEBuilder HiFi mastermix	10 uL
2	pAH09-C2 PCR product (Insert)	0.8 uL
3	pAH05-C10 RD product (Vector)	2 uL
4	H2O	7.2 uL
5	total	20 uL

Assembly mix was kept at 50 C for 60 minutes in a thermocycler. 2 uL of the reaction was used to transform into TOP10 chemically competent cells according to the attached protocol.

WEDNESDAY, 9/14

Colony PCR was performed to verify if the transformants contaided the correct insert. 5 colonies were tested and positive contraol was done from pAH05 and negative control from pUG6.

Following reaction was set up on ice.

Table5

	A	B
1	Dream tag master mix	40 ul
2	forward primer (EK25) 10uM	1,6 ul
3	reverse primer (EK28) 10uM	1,6 ul
4	water	36,8 ul
5	template	

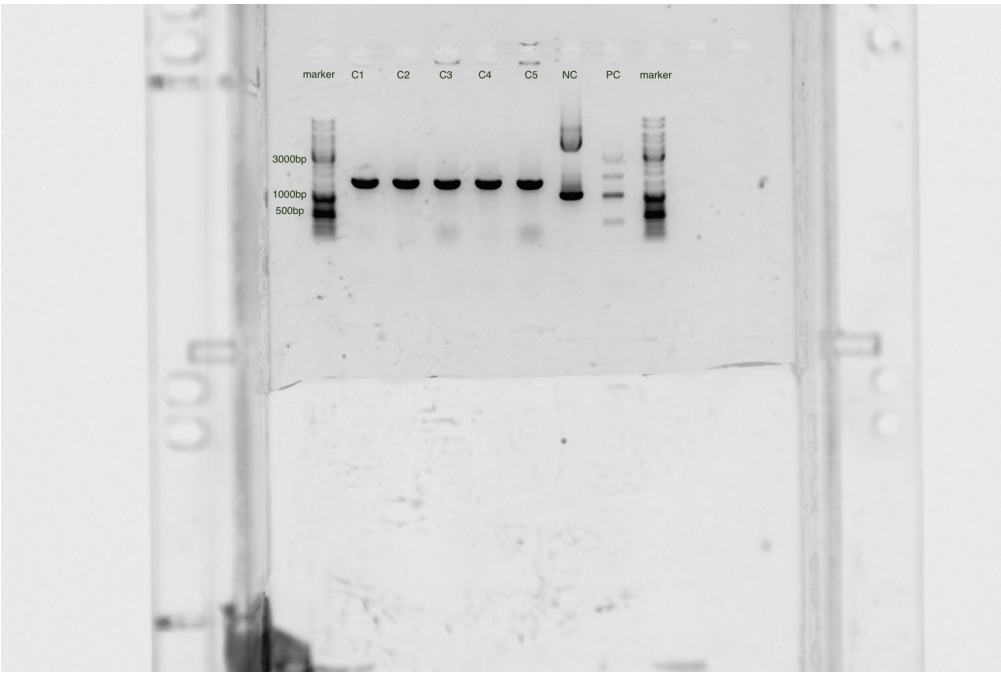
Following cycling conditions were used.

Table6

	A	B	C	D
1	Step	T (C)	time	Cycle(s)
2	Step	95	3 min	1
3	Denaturation	95	30 s	30
4	Annealing	50	30 s	30
5	Extension	72	1 min 30 s	30
6	Final extension	72	15 min	1
7		4	∞	

Colony PCR reaction were then run on gel.

14.9.16 colony PCR pAH05+9 gibson.tif



The size of the transporter is 1540bp so all the analysed colonies seem to contain the correct insert.

Liquid cultures were done for pAH09.5-C1 and C2, overnight at 37 C with shaking.

THURSDAY, 9/15

Miniprep was done with Macherey-Nagel Nucleospin Plasmid miniprep kit and attached protocol. Plasmid concentrations were measured with nanorop.

The next step will be transformation of the purified plasmids into yeast.

09/14-15 Transporter plasmid transformtion and promoter transformation in transporter strains

Project: Transporter
Authors: Pihla Savola
Dates: 2016-09-14 to 2016-09-18

WEDNESDAY, 9/14

This entry describes the transformation of pAH09.5 (QDRI_Cstrep transporter under GAL1 promtoer in pRS415 plasmid) into yeast (SS328-leu).
Also, the stess promoters (pAH01-3) were transformed into SS328-leu that already contains the QDR2 transporter; transformation was done into 3 yeast strain variants - QDR2I (pAH06), QDR2L (pAH07) and QDR2I_Cstrep (pAH09). This promoter+transporter strain will hopefully allow MC induction of the stress promoters.

Liquid cultures were made in 5ml of YPD from following strains:
-pAH06-C2-C7
-pAH07-C1-C4
-pAH09-C2-C5
-SS328-leu

They were left to grow O/N on shaking in 30°C.

THURSDAY, 9/15

ODs from liquid cultures were measured and diluted to reach an OD of about 0,5.

Table1

	A	B	C	D	E	F
1		OD600 (1:10)	real OD	cells+media (ml)		
2	pAH06-C2-C7 (1)	0,716	7,16	0,4+4,6		
3	pAH06-C2-C7 (2)	0,444	4,44	0,6+4,4		
4	pAH07-C1-C4 (1)	0,712	7,12	0,4+4,6		
5	pAH07-C1-C4 (2)	0,742	7,42	0,4+4,6		
6	pAH09-C2-C5 (1)	0,772	7,72	0,4+4,6		
7	pAH09-C2-C5 (2)	0,604	6,04	0,5+4,5		
8	SS328-leu	0,893	8,93	0,3+4,7		

After dilution samples were left to grow for 3h (so that OD reached 1,5) and then competent cells were prepared according the protocol.

Promoter constructs pAH01, pAH02, pAH03 and pAH04 were transformed to strains pAH06, pAH07 and pAH09. Transporter under galactose promoter construct pAH9.5 was transformed into strain SS328-leu. All transformation were done according the LiAc transformation protocol.

SUNDAY, 9/18

Colonies were observed on all the plates. Plates were stored in +4 C.

09/29 Colorimetric Catalase Assay

Project: Transporter
Authors: Pihla Savola
Dates: 2016-09-28 to 2016-10-11

WEDNESDAY, 9/28

Because our foam-based catalase assay didn't manage to differentiate consistently between different MC concentrations, we decided to try a colorimetric catalase assay, hoping for more accuracy. The sample preparation is based on (Valerio, E., Vilares, A., Campos, A., Pereira, P., Vasconcelos, V., (2014) Effects of microcystin-LR on Saccharomyces cerevisiae growth, oxidative stress and apoptosis, Toxicon, 90, 191-198), and the assay kit and protocol itsel are from Abcam.

15 mL precultures of SS328-leu, VL3 and pAH09-C2-C5 in SS328-leu were prepared into YPD by inoculating a single colony into YPD medium in 250 mL flasks. Cultures were grown at shaking (250 rpm), ~25 C.

THURSDAY, 9/29

5ug/ml, 0,5ug/ml and 0,05ug/ml dilutions were made from microcystin-LR extract containing 50 ug/l of toxin.

Only VL3 had grown so we decided to do preliminary catalase activity measurements only for it.

OD600 wes measured from preculture and it was diluted to OD600=0,05 with fresh YPD. Three new cultures were made from preculture: one for each MC concentration. Microcystins were added to cell cultures to reach concentrations 0ug/l, 1 ug/l and 1000ug/l. 1ug/l was made by pipetting 0,4ml of 0,05 ug/ml solution to culture and 1000 ug/l by pipetting 0,4 ml of 50 ug/l solution.

Cells were left to incubator for 4h (≈23°C, 230rpm). After this ODs were measured and volume correspoding to OD=0,2 was pipetted into eppendorf tube.

Table1

	A	B	C
1	c(MC)	OD600	harvested amount (ml)
2	0	0,177	1,13
3	1	0,164	1,09
4	1000	0,257	0,78

Harvested cells were spun down and washed with ice cold PBS. After wash, cells were suspended into 200ul of catalase assay buffer. Cell lysis was performed by adding acid washed glass beads about half of the sample volume, vortexing in +7°C and then centrifuged at 10000g (+4°C) for 15mins. Supernatant was then collected into clean tube.

20mM H_2O_2 standard for catase standard curve was prepared by diluting 1 ul of 0,884M H_2O_2 with 43 ul of ddH_2O_2. 0,5 ml of 1mM H_2O_2 solution was prepared by taking 25ul of 20mM H_2O_2 and diluting it with 475ul of ddH_2O_2.
Standard curve was prepared according the following table:

Table2

	A	B	C	D	E
1	standard	volume of the standard	assay buffer	final volume in well	end CAT/well (mmol)
2	1	0	135	90	0
3	2	3	132	90	2
4	3	6	129	90	4
5	4	9	126	90	6
6	5	12	123	90	8
7	6	15	120	90	10
8	7	7.5	127.5	90	100

Stadard curve samples were pipetted into wells of 96-well plate.

In reaction wells 50ul of cell sample was added and volume was adjusted to 78 ul with assay buffer. Also sample high controls were made. These were replicates of sample wells. 10 ul stop solution was added to sample high control wells and and standard curve wells and mixed. Plate was incubated at 25°C for 5 mins.

12 ul fresh 1 mM H2O2 was added into each well of both samples and sample HCs to start the reaction and incubated at 25°C for 30mins. 10 ul of stop solution was added into each sample and positive control wells to stop the reaction.

Development mix was prepared for 15 samples (+1 extra) according the following table:

Table3

	A	B	C
1	componenet	colorimetric reaction mix (ul)	
2	Assay buffer	736	
3	OxiRed Probe	32	
4	HRP solution	32	

50ul of development mix was added to each well and mixed. Plate was incubated 10mins at 25°C and then fluorescence was measured at 570nm.

Order in 96-well plate:

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	stand_1	stand_2	stand_3	stand_4	stand_5	stand_6	stand_7					
B	VL3_0	HC VL3_O	VL3_1	HC VL3_1	VL3_1000	HC VL3_100O						
C	PC	0,5V VL3_0	0,5V VL3_1									
D												
E												
F												
G												
H												

0,5V (half-volume) samples were tests done with half of the volume for each component.

Results (OD570):

Table8

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	12
2	A	0.111	0.267	0.518	0.58	0.726	1.069	0.388					
3	B	1.969	2.083	1.094	1.945	2.246	1.875						
4	C	0.114	1.315	1.596									
5	D												
6	E												
7	F												
8	G												
9	H												

Standard curve was plotted according to instructions of the assay kit manual:

Table9

	A	B	C
1	Standard #	H2O2 (nmol)	OD570
2	1	0	0.111
3	2	0.2	0.267
4	3	0.4	0.518
5	4	0.6	0.58
6	5	0.8	0.726
7	6	1	1.069

The standard curve was plotted with the intercept forced to 0, as otherwise small OD570 readings would correspond to negative H2O2 amounts.

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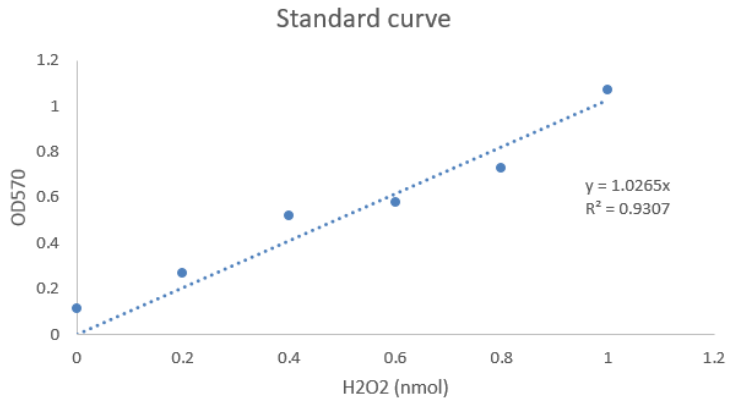

OD570 signal changes (high control, where no H2O2 is degraded, and the sample, where catalase is allowed to act) were calculated, and the corresponding amount of decomposed H2O2 was read from the standard curve. Catalase activiity (decomposed H2O2/(reaction time in minutes * pretreated sample volume (mL) added into the reaction well) was then calculated, with reaction time 30 min and sample volume 0.050 mL.

Table10

	A	B	C	D
1	c(MC, ug/L)	Signal changes (HC-sample)	Decomposed H2O2	Catalase activty (mU/mL)
2	0	0.114	0.11105699	0.0740379933
3	1	0.851	0.829030687	0.5526871247
4	1000	-0.371	-0.361422309	-0.240948206

Something seems to have gone wrong with the 1000 ug/L sample since the activity is negative, but the catalase activity of the other two samples is shown below:

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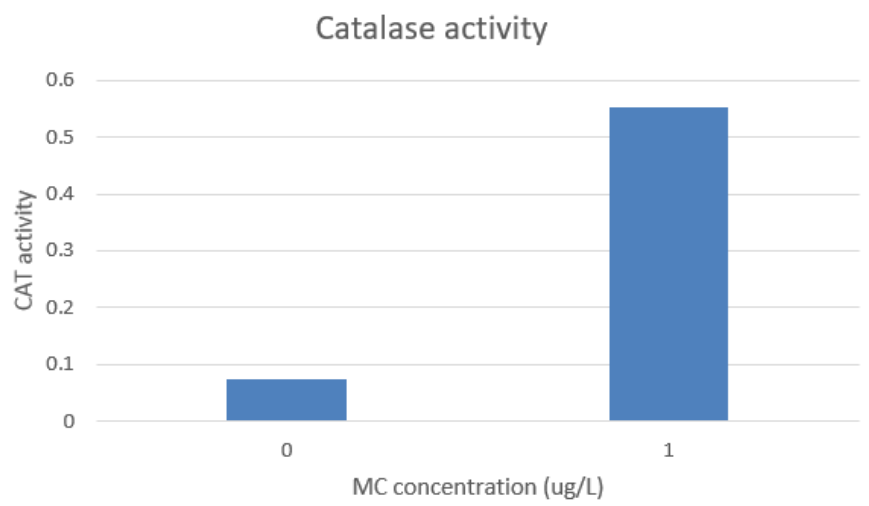

It was also decided based on the tested 0.5V samples that half the volume can't be easily multiplied back to the normal volume, so it looks like it's better to use the kit specification volume since there seems to be a difference.

MONDAY, 10/3

5 mL precultures of SS328-leu, VL3 and pAH09-C2-C5 in SS328-leu were prepared into YPD by inoculating a single colony into YPD medium in 50 mL falcon tubes. Cultures were grown at shaking (250 rpm), 20° C.

TUESDAY, 10/11

5ug/ml, 0,5ug/ml and 0,05ug/ml dilutions were made from microcystin-LR extract containing 50 ug/l of toxin.

OD600 wes measured from precultures and they were diluted to OD600=0,05 with fresh YPD. Three new cultures were made from each of the precultures: one for each MC concentration. Microcystins were added to cell cultures to reach concentrations 0ug/l, 1 ug/l and 1000ug/l. 1ug/l was made by pipetting 0,4ml of 0,05 ug/ml solution to culture and 1000 ug/l by pipetting 0,4 ml of 50 ug/l solution.

Cells were left to incubator for 4h (20°C, 230rpm). After this ODs were measured and volume correspoding to OD=0,2 was pipetted into eppendorf tube.

Table4

	A	B	C	D
1	strain	c(MC)	OD600	harvested amount (ml)
2	VL3	0	0.355	2.25
3		1	0.139	5.75
4		1000	0.208	3.846
5	SS328-leu	0	0.088	9.09
6		1	0.084	9.52
7		1000	0.175	4.57
8	pAH09	0	0.28	2.8
9		1	0.156	5.13
10		1000	0.23	3.48

Harvested cells were spun down and washed with PBS. After wash, cells were suspended into 200ul of catalase assay buffer. Cell lysis was performed by adding acid washed glass beads about half of the sample volume, vortexing in +7°C for 10 min and then centrifuged at 10 000g (+4°C) for 15mins. Supernatant was then collected into clean tube.

20mM H2O2 standard for catase standard curve was prepared by diluting 5 ul of 0,884M H2O2 with 215 ul of ddH2O2. 1mM H2O2 solution was prepared by taking 50ul of 20mM H2O2 and diluting it with 950ul of ddH2O2.
Standard curve was prepared according the following table:

Table5

	A	B	C	D	E
1	standard	volume of the standard	assay buffer	final volume in well	end CAT/well (mmol)
2	1	0	270	90	0
3	2	6	264	90	2
4	3	12	258	90	4
5	4	18	252	90	6
6	5	24	246	90	8
7	6	30	240	90	10
8					

Stadard curve samples were pipetted into wells of a 96-well plate.

In reaction wells 20ul of cell sample was added and volume was adjusted to 78 ul with assay buffer. Also sample high controls were made. These were replicates of sample wells. 10ul stop solution was added to sample high control wells and and standard curve wells and mixed. Plate was incubated at 25°C for 5 mins.

12 ul fresh 1 mM H2O2 was added into each well of both samples and sample HCs to start the reaction and incubated at 25°C for 30mins. 10 ul of stop solution was added into each sample and positive control wells to stop the reaction.

Development mix was prepared for 50 reactions according the following table:

Table6

	A	B	C
1	componenet	colorimetric reaction mix (ul)	
2	Assay buffer	2300	
3	OxiRed Probe	100	
4	HRP solution	100	

50ul of development mix was added to each well and mixed. Plate was incubated 10mins at 25°C and then fluorescence was measured at 570nm.

Order in 96-well plate:

Table7

	1	2	3	4	5	6	7	8	9	10	11
A											
B		stand_7	stand_7	VL3_1000	HC VL3_0	SS_1000	HC SS_1000	pAH_1000	HC pAH_1000		
C		stand_6	stand_6	VL3_1000	HC VL3_0	SS_1000	HC SS_1000	pAH_1000	HC pAH_1000		
D		stand_5	stand_5	VL3_1	HC VL3_1	SS_1	HC SS_1	pAH_1	HC pAH_1	PC (with H2O2)	
E		stand_4	stand_4	VL3_1	HC VL3_1	SS_1	HC SS_1	pAH_1	HC pAH_1	PC	
F		stand_3	stand_3	VL3_0	HC VL3_1000	SS_0	HC SS_0	pAH_0	HC pAH_0		
G		stand_2	stand_2	VL3_0	HC VL3_1000	SS_0	HC SS_0	pAH_0	HC pAH_0		
H											

It was noticed when measuring the catalase activty that there was a lot of foam in some of the sample wells; this can have a big effect on the reading. Because of this, measurements were done multiple times, as the foam degraded with time. It might be possible that the color itself was also affected by the wait. However, it was clearly noted that the readings dropped significantly when the foam degraded (e.g. the PC well)

OD570 right after incubation:

Table11

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	12
2	A												
3	B		0.874	0.846	1.243	1.317	1.245	1.203	1.263	1.166			
4	C		0.72	0.687	1.222	1.322	1.24	1.3	1.261	1.174			
5	D		0.521	0.538	1.196	0.043	1.277	1.249	1.182	1.255	0.536		
6	E		0.423	0.417	1.32	1.287	1.214	1.347	1.18	1.282	0.847		
7	F		0.211	0.214	1.199	1.384	1.165	1.247	1.162	1.347			
8	G		0.048	0.049	1.276	1.199	1.354	1.279	1.146	1.322			
9	H												

5 min after incubation:

Table12

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	
2	A												
3	B		0.862	0.845	1.191	1.311	1.244	1.203	1.26	1.165			
4	C		0.719	0.687	1.219	1.321	1.238	1.299	1.26	1.169			
5	D		0.521	0.538	1.194	0.043	1.275	1.246	1.178	1.252	0.524		
6	E		0.422	0.417	1.321	1.28	1.213	1.345	1.179	1.28	0.673		
7	F		0.212	0.215	1.199	1.38	1.163	1.22	1.142	1.341			
8	G		0.049	0.049	1.275	1.197	1.352	1.276	1.117	1.317			
9	H												

13 min after incubation

Table13

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	12
2	A												
3	B		0.857	0.844	1.167	1.255	1.224	1.199	1.209	1.161			
4	C		0.717	0.685	1.152	1.275	1.23	1.292	1.238	1.141			
5	D		0.523	0.539	1.161	0.042	1.242	1.193	1.13	1.23	0.476		
6	E		0.423	0.418	1.314	1.266	1.198	1.284	1.173	1.215	0.05		
7	F		0.215	0.217	1.196	1.365	1.138	1.211	1.138	1.302			
8	G		0.049	0.049	1.266	1.189	1.344	1.267	1.111	1.303			
9	H												

17 min after incubation

Table14

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	
2	A												
3	B		0.83	0.846	1.129	1.245	1.213	1.199	1.209	1.159			
4	C		0.717	0.68	1.14	1.266	1.218	1.291	1.238	1.122			
5	D		0.525	0.541	1.161	0.042	1.232	1.188	1.118	1.228	0.464		
6	E		0.424	0.419	1.312	1.248	1.195	1.285	1.16	1.216	0.043		
7	F		0.217	0.219	1.195	1.324	1.127	1.197	1.129	1.297			
8	G		0.049	0.05	1.25	1.179	1.328	1.246	1.092	1.257			
9	H												

23 min after incubation:

Table15

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		1	2	3	4	5	6	7	8	9	10	11	12
2	A												
3	B		0.83	0.846	1.129	1.244	1.212	1.198	1.208	1.159			
4	C		0.716	0.681	1.14	1.266	1.216	1.288	1.236	1.121			
5	D		0.526	0.542	1.157	0.043	1.228	1.184	1.112	1.224	0.464		
6	E		0.424	0.42	1.308	1.243	1.196	1.283	1.158	1.214	0.043		
7	F		0.218	0.22	1.191	1.319	1.123	1.194	1.128	1.29			
8	G		0.049	0.05	1.245	1.176	1.325	1.243	1.091	1.254			
9	H												

33 min after incubation:

Table16

	A	B	C	D	E	F	G	H	I	J	K	L	
1		1	2	3	4	5	6	7	8	9	10	11	
2	A												
3	B		0.83	0.846	1.126	1.24	1.209	1.202	1.206	1.156			
4	C		0.716	0.681	1.14	1.265	1.216	1.285	1.234	1.12			
5	D		0.528	0.543	1.155	0.047	1.223	1.182	1.107	1.22	0.469		
6	E		0.425	0.421	1.304	1.237	1.186	1.282	1.157	1.214	0.045		
7	F		0.22	0.222	1.188	1.314	1.121	1.193	1.129	1.281			
8	G		0.051	0.05	1.24	1.174	1.323	1.24	1.092	1.249			
9	H												

Standard curve was plotted according to instructions of the assay kit manual:

Table17

	A	B	C
1	Standard #	H2O2 (nmol)	OD570 (average of 2 samples)
2	6	10	0.838
3	5	8	0.6985
4	4	6	0.533
5	3	4	0.4215
6	2	2	0.218
7	1	0	0.0495

The standard curve was plotted with the intercept forced to 0, as otherwise small OD570 readings would correspond to negative H2O2 amounts.

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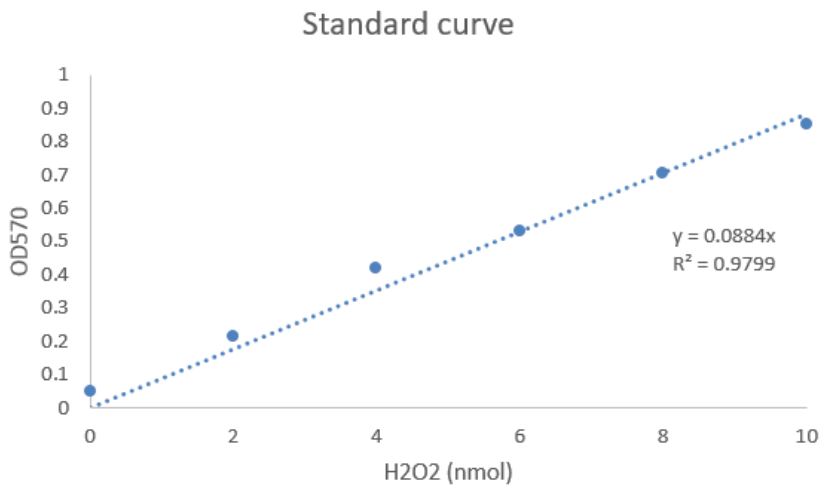

OD570 signal changes (high control, where no H2O2 is degraded, and the sample, where catalase is allowed to act) were calculated from the 17 min read as there was little foam left at that point and the reads didn't change much anymore. The corresponding amount of decomposed H2O2 was read from the standard curve. Catalase activiity (decomposed H2O2/(reaction time in minutes * pretreated sample volume (mL) added into the reaction well) was then calculated, with reaction time 30 min and sample volume 0.020 mL.

Table18

	A	B	C	D	E	F	G
1	Strain	c(MC, ug/L)	Sample, average	Sample HC, average	Signal changes (HC-sample)	Decomposed H2O2	Catalase a (mU/mL)
2	VL3	0	1.2225	1.2515	0.029	0.3280542986	0.54675
3		1	1.2365	1.248	0.0115	0.1300904977	0.21681
4		1000	1.1345	1.2555	0.121	1.3687782805	2.28129
5	SS328-leu	0	1.2275	1.2215	-0.006	-0.0678733032	-0.11312
6		1	1.2135	1.2365	0.023	0.2601809955	0.43363
7		1000	1.2155	1.245	0.0295	0.3337104072	0.55618
8	pAH09-C2-C5	0	1.1105	1.277	0.1665	1.8834841629	3.13914
9		1	1.139	1.222	0.083	0.9389140271	1.56485
10		1000	1.2235	1.1405	-0.083	-0.938914027	-1.56485

Catalase activities are plotted below:

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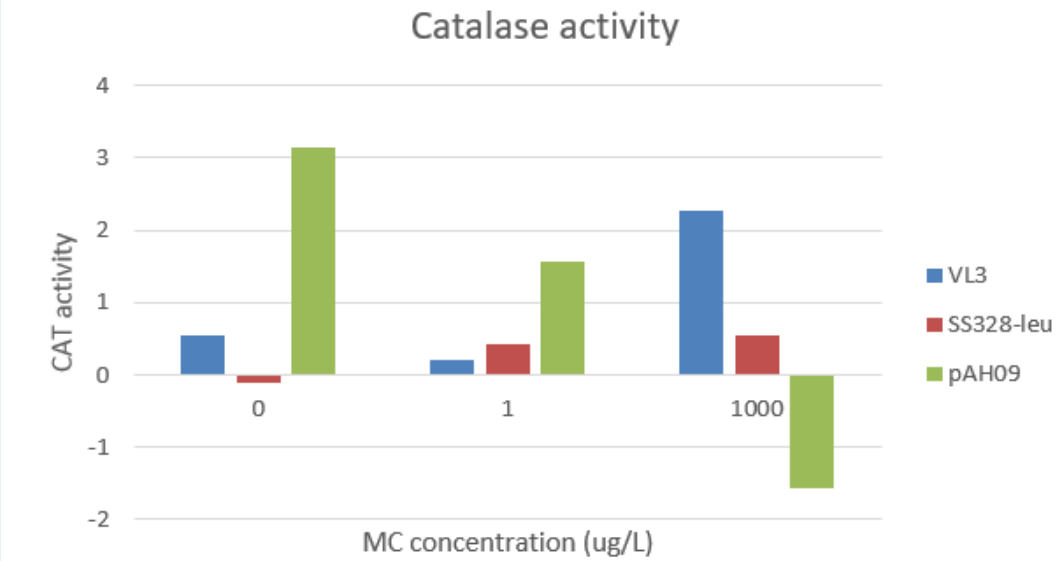

It is clear however that the results hardly tell anything (huge margin of error) as the there's negative activities, and e.g. the activities for VL3 are entirely diferent from last measurement (last measurement, there was a big activity increase from concentration 0 to 1). Possible things that contribute to the inaccuracy and failure of the experiment are

- the different growth rates of the different samples (samples not at the same stage of growth maybe, so activities not comparable - although this doens't explain negative values)
- Inaccurate way of harvesting cells for activity measurement - multiple steps of spinning culture down and removing supernatant, it's likely that when removing supernatant, some cells have been also taken with it.
- Unsuitability of lysis procedure, the assay is desinged for mammalian cells and in the protocol, homogenization of cells is done with just up/down pipetting, and although the kit specification says that the kit could be used for other kinds of cells also, perhaps the lysis wasn't so suitable.
- Foaming of samples and delay in measurement of color

- Pipetting errors and pipetting inaccuracy.
- Possibly even smaller cell amounts would in the wells would be good.

This assay could likely give quite accurate activities, but clearly it requires a lot more optimization, for which there isn't enough time. In this new light, our old foam test wasn't that bad after all, since no assay seems to be easy and accurate without a lot of optimization.